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(54) **Title:** USE OF AN ANTAGONIST OF EPAC FOR TREATING HUMAN CARDIAC HYPERTROPHY

(57) **Abstract:** The present invention relates to the use of at least one Epac (Exchange Protein directly Activated by cAMP) antagonist for the manufacture of a medicament intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis.

USE OF AN ANTAGONIST OF EPAC FOR TREATING HUMAN CARDIAC HYPERTROPHY

FIELD OF THE INVENTION

The present invention relates to methods and compositions for treating Human Cardiac Hypertrophy (HCM). More specifically, the present invention relates to the use of an antagonist of Epac for treating HCM.

BACKGROUND

Physiologic hypertrophy, such as an exercise-induced cardiac hypertrophy, represents a favorable adaptive change in the heart that accommodates to the increases in body demand, and does not lead to heart failure. In contrast, pathologic hypertrophy, such as pressure overload-induced hypertrophy, is a maladaptive response to pathologic stimuli that, if not ameliorated, usually leads to heart failure.

Human cardiac hypertrophy (HCM) affects an estimated 600,000 to 1.5 million Americans, or one in 500 people. It is more prevalent than multiple sclerosis, which affects one in 700 people. HCM is the most common cause of sudden cardiac death in people under age 30.

Human cardiac hypertrophy (HCM) often develops as a by-product of hypertension or valvular heart disease. Adult cardiomyocytes are unable to divide and respond to stress and growth stimuli by increasing their rate of protein synthesis, resulting in increased cell volume (Sadoshima et al. 1997). Growth of individual cardiomyocytes results in thickening of the heart. Cardiac hypertrophy is a potent risk factor for the development of cardiac arrhythmias, diastolic dysfunction, congestive heart failure, and death. (Hennersdorf et al. 2001; Vakili et al. 2001).

To date a variety of drug treatments are currently used for treating human cardiac hypertrophy.

For instance beta-blocking drugs slow the heart beat and reduce its force of contraction. These drugs usually relieve chest pain, breathlessness and palpitation, but occasionally excessive heart rate slowing with these drugs can cause fatigue.

The second major group of drugs used are the calcium antagonists or calcium channel blockers. Within this group verapamil is the drug which has been most commonly used in HCM. It improves the filling of the heart and like beta-blockers, reduces symptoms such as

chest pain, breathlessness and palpitations. Also, like beta-blockers, verapamil can cause excessive slowing of the heart rate and lower blood pressure.

Anti-arrhythmic drugs have been also used for treating of cardiac hypertrophy. These drugs might be used when an arrhythmia such as tachycardia is detected and felt to be important in an individual case. Of these anti-arrhythmic drugs, Amiodarone (Cordarone) is the most commonly used in hypertrophic cardiomyopathy. It is a powerful and effective drug. But it does have several potentially serious side effects, including pulmonary toxicity (2%-7% in some studies, but as high as 10%-17% in some reports) liver function test abnormalities (4%-9%), hyperthyroidism (about 2%), and hypothyroidism (2%-4% in some cases, but as high as 8%-19% in some series), proarrhythmia (2%-5%) and optic neuropathy, which can lead to blindness.

Thus, there is a permanent need in the art to find alternative drugs for treating HCM that preferably do not show the side effects as described above.

It is now well admitted that Ca2+-sensitive signaling pathways play crucial roles in cardiomyocyte hypertrophy (Frey et al, 2000). Two prominent Ca2+-dependent pathways involve the Ser/Thr protein phosphatase calcineurin (Liang et al, 2003; Molkentin, 2004) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Zhang & Brown, 2004). Activation of calcineurin by Ca2+ results in the dephosphorylation and nuclear translocation of cytoplasmic nuclear factor of activated T cells (NFAT) transcription factors which then upregulate transcription of hypertrophic genes. Calcineurin can also relieve the inhibition of class II histone deacetylases (HDACs) on the myocyte enhancer factor 2 (MEF2), thereby allowing this transcription factor to induce hypertrophic gene expression. In addition, CaMKII is known to activate MEF2 upon HDACs phosphorylation (McKinsey & Olson, 2004). To date, the participation of small G proteins of the Rho family in the regulation of these hypertophic signaling cascades is not well defined.

Among the superfamily of small G proteins, the Rho family which includes Rho, Rac and Cdc42 has attracted much interest for they have been shown to play key roles in the generation of cytoskeletal structures (Hall, 1998). Indeed, Rho is important for the formation of stress fibers and focal adhesions in fibroblasts, whereas Rac and Cdc42 are involved in the regulation of more dynamic structures such as membrane ruffles, lamellipodia and filopodia (Hall, 1998). Several studies have pointed out the role of Rho proteins in the development of cardiomyocyte hypertrophy (Clerk & Sugden, 2000). For instance, two potent hypertrophic stimuli, endothelin 1 (ET-1) and phenylephrine (PE) induce rapid activation of endogenous Rac in neonatal cardiomyocytes (Clerk et al, 2001). In addition, adenoviral infection of

cardiomyocytes with a constitutive active form of Rac (RacG12V) also increases ANF expression and protein synthesis, and promotes morphological changes associated with myocyte hypertrophy (Pracyk et al, 1998). *In vivo* evidence for the role of Rho proteins in cardiac hypertrophy came from transgenic mice specifically expressing RacG12V in the heart. These mice develop a dilated cardiomyopathy associated with deregulation of cardiomyocyte focal adhesions (Sussman et al, 2000). These data suggest that Rho proteins, especially Rac control hypertrophic response and are likely to be involved in cardiac remodelling, and the pathogenesis of cardiomyopathy characterized by cellular enlargement.

Cyclic adenosine 3',5'-monophosphate (cAMP) is one of the most important second messenger in the heart because it regulates many physiological processes such as cardiac contractility, relaxation and automaticity. Classically, these effects are attributed to activation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and protein kinase A (PKA) by cAMP' (Fimia & Sassone-Corsi, 2001). PKA is considered to be the essential effector molecule mediating the many physiological effects of Gs-coupled-receptors. A classical example is the stimulation of cardiac β-adrenergic receptors in which PKA phosphorylates several key proteins involved in excitation-contraction coupling, such as L-type Ca^{2+} channels, phospholamban, ryanodine receptors (RyR) and troponin I (Bers & Ziolo, 2001).

The recent discovery of Epac (exchange proteins directly activated by cAMP) as proteins which are directly activated by cAMP has broken the dogma surrounding cAMP and PKA (patent US 6,987,004 ; Bos, 2003; de Rooij et al, 1998; Kawasaki et al, 1998). Epac proteins are guanine nucleotide exchange factors (GEFs) that bind cAMP with affinities similar to that of the regulatory subunit of PKA (de Rooij et al, 1998; Kawasaki et al, 1998). They have been shown to function as GEFs for the Ras-like small GTPases Rap1 and Rap2 and are directly activated by cAMP in a PKA independent manner (Bos, 2003). There are two isoforms of Epac, Epac 1 and Epac 2 both consisting of a regulatory and a catalytic region (de Rooij et al, 1998; Kawasaki et al, 1998). Epac1 is highly expressed in the heart (Kawasaki et al, 1998). Epac 2 has an additional cAMP binding domain which is dispensable for cAMP-induced Rap activation (de Rooij et al, 2000). Following cAMP binding, Epac catalyses the exchange of GDP for a GTP of the small GTPases Rap, allowing interaction with their target effectors (Rehmann et al, 2003). Recent studies indicate that Epac is involved in cell adhesion (Enserink et al, 2004; Rangarajan et al, 2003), neurite extension (Christensen et al, 2003), and regulates the amyloid precursor protein and insulin secretion (Maillet et al, 2003; Ozaki et al, 2000). A recent study has provided experimental evidence that Epac1 stimulates the activity

of the small GTPase, Rac in a cAMP- dependent but PKA- independent manner in neuronal cells (Maillet et al, 2003).

SUMMARY

The Inventors now surprisingly show that Epac1 stimulates the activity of the small GTPase, Rac and increases the expression of hypertrophic gene markers in cultured cardiomyocytes. Furthermore, the Inventors demonstrate that Epac1 induces cardiomyocyte hypertrophy. This process is associated with intracellular [Ca2+] mobilization and the activation of the transcription factors NFAT and MEF2. Altogether, these findings identify the cAMP-binding protein, Epac as a new positive regulator of cardiac growth.

A first aspect of this invention thus resides in the use of an antagonist of Epac for the manufacture of a medicament for treating cardiac hypertrophy, and especially, human cardiac hypertrophy.

A further object of this invention resides in a method for treating cardiac hypertrophy, especially human cardiac hypertrophy, comprising administering at least one antagonist of Epac to the subject.

A further object of the invention resides in a pharmaceutical composition useful for treating cardiac hypertrophy, and especially human cardiac hypertrophy, comprising at least one antagonist of Epac.

In one embodiment, the antagonist of Epac is a compound natural or not that impedes or limits the activation of Epac by cAMP or hypertrophic stimuli such as angiotensin II, endothelin I and phenylephrine. In a particular embodiment, said antagonist is a cAMP analog, that competes with cAMP for binding to Epac, but to thereupon either block or significantly inhibit the biological response induced by cAMP or hypertrophic stimuli such as angiotensin II, endothelin I and phenylephrine. cAMP analog candidates are those compounds that have an affinity for binding to Epac that is either not significantly different from, or higher than cAMP, and that induce no, or a significantly lower level of biological response than native cAMP.

In a further embodiment, the antagonist of Epac, is a compound that is able to inhibit the expression of Epac. In a particular embodiment, said antagonist is an antisense oligonucleotide, a siRNA or a ribozyme.

A further object of the invention resides in a method for screening a compound, and especially a cAMP analog, that affects the activation of Epac.

In one embodiment, the screening method involves the steps of:

- a) incubating Epac with i) at least one effector of Epac, chosen among, but not limited to, Rap1, Rap2, Rac or Ras, ii) cAMP and iii) said compound to be tested,
- b) assessing the activation of said effector by Epac,

wherein a lower activation, in comparison with the activation provided in absence of said compound to be tested, indicates that said compound is an antagonist of Epac. The assay of effector activation could be performed as hereunder described in Example 1 "Rac activation assay", ie wherein said effector is coupled in frame with GST. In a further embodiment said effector is Rap1 that can be loaded with fluorescently labelled 2', 3'-bis(O)-N-methylanthraniloyl-guanosinediphosphate (mGDP). In this particular embodiment, the activation of Rap1 is assessed by detecting in real time the release of mGDP with a fluorescence spectrometer as previously described (Van den Berghe et al., 1997).

In a further embodiment, the screening method involves the step of :

a) providing cardiomyocytes that either constitutively expressed an activated form of Epac or Epac wild type, or have been treated with hypertrophic stimuli such as angiotensin II, endothelin I and phenylephrine.

b) exposing said cardiomyocyte to one of the compounds to be tested

c) assessing the effects of said compound on the hypertrophy properties of said cardiomyocytes

wherein the inhibition of the hypertrophic properties indicates that said compound is an antagonist of Epac.

In a particular embodiment, cardiomyocyte hypertrophy properties are determined by measuring cell size, protein content and/or the expression of mRNA coding for hypertrophic gene markers such as the natriuretic factor or NFAT transcriptional activity.

In a further embodiment, the screening method can result in the combination of the two above described methods.

A further object of the invention resides in a non human model of cardiac hypertrophy wherein cardiac hypertrophy has been induced by the specific expression of a positive dominant form of Epac, and especially Epac1. In a particular embodiment, said model is a mouse. In another particular embodiment, said positive dominant form of Epac is under the control of a cardiac specific promoter such as α-Myosin Heavy Chain promoter. In a further embodiment the positive dominant form of Epac1 result in Epac-ΔcAMP which contains a deletion of the first 322 amino acids of Epac1 (de Rooij et al., 1998).

In one embodiment, Epac is the protein shown in SEQ ID NO: 2 (Epac1, Accession: NM_006105) or SEQ ID NO: 4 (Epac2, Accession: NM_007023), or a partial protein thereof, or an ester, amide or salt thereof.

DETAILED DESCRIPTION

The present invention relates to the use of at least one Epac (Exchange Protein directly Activated by cAMP) antagonist for the manufacture of a medicament intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis.

An antagonist of Epac is herein defined as a compound that is able to inhibit the expression, the activation or the activity of Epac.

An antagonist of Epac that inhibits the expression of Epac means that said antagonist impedes the process of transcription of the Epac gene and / or of the translation of the mRNA of Epac into the Epac protein.

An antagonist of Epac that inhibits the activation of Epac means that said antagonist impedes the activators of Epac to activate Epac.

An antagonist of Epac that inhibits the activity of Epac means that said antagonist impedes Epac function. Such an antagonist may block the catalysis by Epac of the exchange of GDP for a GTP.

The invention relates in particular to the use as defined above, wherein the antagonist is selected from the list comprising Epac activation inhibitors, Epac activity inhibitors, Epac intracellular localization disruption agents, and Epac expression inhibitors.

An "Epac expression inhibitor" impedes or decreases the expression of the protein Epac. Said inhibitor may block or decrease the transcription and / or the translation process of Epac. The inhibition of Epac expression can be assessed by Western blot or reverse transcriptase polymerase chain reaction.

An "Epac activation inhibitor" impedes the activation of Epac, particularly by the activators of Epac such as cAMP. The inhibition of Epac activation can be assessed by measuring the GTP form of its effector, Rap1 or Rap2, using pull down assay as described in Maillet et al., 2003.

An "Epac activity inhibitor" is a compound which impedes the normal function of Epac, such as the exchange of GDP for a GTP or the activation of the c-Jun NH2-terminal kinase.

The inhibition of Epac activity can be assessed by measuring either the GTP form of its effector, Rap1 or Rap2 using pull down assay or the activation of c-Jun NH2-terminal kinase using a kinase assay as described in Hochbaum et al. (2003).

An "Epac intracellular localization disruption agent" is a compound which impedes the proper cellular localization of Epac. Epac cellular distribution can be assessed by immunocytochemistry using Epac selective antibodies.

The invention further relates to the use as defined above, wherein the antagonist is an Epac activation inhibitor selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against Epac cAMP binding sites,
- cAMP analogues, such as cAMP derivatives or 8-(4-chlorophenylthio)-2'-O-methyladenosine-3'-5-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) derivatives,
- Brefeldin A or derivatives thereof.

Compounds capable of inhibiting the activation of Epac include in particular those able to interact with natural agonists of Epac, such as cAMP, and /or to interact in the binding of said agonists to Epac, and / or to inhibit the activation of Epac resulting from said binding.

The term "agonist of Epac" refers to compounds that bind to Epac and activate Epac through this binding.

An inhibitor of activation of Epac may be an antibody or fragment thereof, or an aptamer directed against the Epac cAMP binding sites, thus impeding the binding of said cAMP to Epac.

In the present invention, the term "antibody" refers to a polyclonal or monoclonal antibody. The terms "polyclonal" and "monoclonal" refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to a particular method of production.

A mammal, such as a rabbit, a mouse, or a hamster, can be immunized with an immunogenic form of the protein, such as the entire protein or a part of it. The protein or part of it can be administered in the presence of an adjuvant.

The term "immunogenic" refers to the ability of a molecule to elicit an antibody response. Techniques for conferring immunogenicity to a protein or part of it which is not itself immunogenic include conjugation to carriers or other techniques well known in the art.

The immunization process can be monitored by detection of antibody titers in plasma or serum. Standard immunoassays, such as ELISA can be used with the immunogenic protein or peptide as antigen to assess the levels of antibody.

According to the invention, an antibody which is an activation inhibitor of Epac is directed against Epac cAMP binding sites.

In the present invention, the term "aptamer" refers to a nucleic acid molecule or oligonucleotide sequence that binds specifically to the Epac protein. Aptamers may be DNA or RNA and may include nucleotide derivatives. Aptamers may be a single strand or a double strand of usually 16 to 60 nucleotides long. The method called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) is generally used to select and identify aptamers exhibiting affinity for the target.

According to the invention, an aptamer which is an activation inhibitor of Epac binds specifically to Epac cAMP binding sites.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 12, more preferably at least 15, and still preferably at least 20 nucleotides, preferably no more than 100 nucleotides, still preferably no more than 70 nucleotides, and which is hybridizable to a Epac genomic DNA, cDNA, or mRNA.

An inhibitor of activation of Epac can consist in an analog of cAMP which impedes the binding of said cAMP to Epac. In a particular embodiment, said antagonist is a cAMP analog, that competes with cAMP for binding to Epac, but to thereupon either block or significantly inhibit the biological response induced by cAMP or hypertrophic stimuli such as angiotensin II, endothelin I and phenylephrine. cAMP analog candidates are those compounds that have an affinity for binding to Epac that is either not significantly different from, or higher than cAMP, and that induce no, or a significantly lower level of biological response than native cAMP.

Said analog of cAMP can be identified by the screening methods described hereinafter.

Another inhibitor of Epac activation may be Brefeldin A, a small hydrophobic compound produced by toxic fungi. Brefeldin A is a macrocyclic lactone exhibiting a large range of antibiotic activity. Brefeldin A can bind at the Rap-GDP/Epac interface, thus freezing the complex in an abortive conformation that cannot proceed to nucleotide dissociation.

The invention also relates to the use as defined above, wherein the antagonist is an Epac activity inhibitor, in particular an inhibitor of the Guanine nucleotide Exchange Factor

(GEF) domain of Epac, or an inhibitor of the Ras Exchange Motif (REM) domain of Epac, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against the GEF domain or the REM domain of Epac,
- Brefeldin A or derivatives thereof.

The Guanine nucleotide Exchange Factor domain (GEF) is located into the catalytic region of Epac protein and is involved in Epac catalytic activity. The GEF domain of Epac is located between amino acids 616-848 of Epac1 and 768-1005 of Epac2.

The Ras Exchange Motif (REM) domain is located into the catalytic region of Epac protein and participates to Epac catalytic activity. The REM domain of Epac is located between amino acids 342-466 of Epac1 and 495-615 of Epac2.

The invention also relates to the use as defined above, wherein the antagonist is an Epac intracellular localization disruption agent, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against an Epac cellular localization domain, such as the Dishevelled Egl-10 Pleckstrin (DEP) domain,
- Brefeldin A or derivatives thereof.

The term "Epac cellular localization domain" refers to a domain which is involved in membrane localization. An Epac cellular localization domain is for example the Dishevelled Egl-10 Pleckstrin (DEP) domain.

The invention also relates to the use as defined above, wherein the antagonist is an Epac expression inhibitor selected from the list comprising:

- an antisense nucleic acid directed against Epac mRNAs,
- a single stranded DNA, directed against Epac double strand DNA,
- a double stranded RNA, a siRNA or a shRNA, comprising Epac nucleic acid sequences,
- a ribozyme directed against Epac mRNAs.

Inhibitors of the expression of Epac include for instance antisense oligonucleotides, or interfering RNAsi, or ribozymes, targeting the Epac gene.

The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. A "promoter" or "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated

into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription. In particular, the term gene may be intended for the genomic sequence encoding a protein, i.e. a sequence comprising regulator, promoter, intron and exon sequences.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, i.e., the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

As used herein, the term "Epac gene" denotes the gene encoding for Epac (exchange proteins directly activated by cAMP) of any species, especially human Epac, but also other mammals or vertebrates to which the invention can apply. Unless otherwise indicated, the term "Epac" is used indifferently to designate the Epac gene or the encoded protein Epac throughout the text. There are two isoforms of Epac, Epac 1 (also named Rap guanine nucleotide exchange factor (GEF) 3) and Epac 2 (also named Rap guanine nucleotide exchange factor (GEF) 4) such as described in de Rooij et al, 1998 and Kawasaki et al, 1998. In addition, a related protein named Repac (for related to Epac) has been identified by Ichiba et al. (1999). Repac shows close sequence similarity to Epac 2 and lacks the regulatory sequences present in Epac1 and Epac2 (Ichiba et al., 1999). Homo sapiens Epac 1 gene is localized on chromosome 12. The nucleotide and amino acids sequences of Epac1, SEQ ID N0 : 1 and 2 respectively, are deposited in Genebank under accession number NM_006105. The nucleotide and amino acids sequences of Epac 2, SEQ ID N0 : 3 and 4 respectively, are deposited in Genebank under accession number NM_007023. Epac 2 is mapped to human chromosome 2q31. The nucleotide and amino acids sequences of Repac, SEQ ID N0 : 5 and 6 respectively, are deposited in Genebank under accession number BC039203. Repac is mapped to human chromosome 7p15.3.

Antisense molecules can be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in inhibition of peptide synthesis. The antisense oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules can also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm. In addition, antisense deoxyoligoribonucleotides can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which

digests the target RNA in the duplex. Antisense DNA can be expressed via the use of a single stranded DNA intracellular expression vector or equivalents and variations thereof.

Antisense nucleic acids are useful for regulating and controlling the expression of the Epac protein gene *in vivo* and *in vitro*, and are also useful for the treatment of the diseases disclosed above. Technologies related to such antisense RNAs and gene therapies are known to the skilled man. The novel antisense oligonucleotides complementary to any sequence of the human Epac RNA, which according to the broadest definition can be of a length ranging from 7 to 40 nucleotides, have preferably a length ranging from 15 to 25 nucleotides, most preferably about 20 nucleotides.

The term "complementary" means that the antisense oligonucleotide sequence can form hydrogen bonds with the target mRNA sequence by Watson-Crick or other base-pair interactions. The term shall be understood to cover also sequences which are not 100 % complementary. It is believed that lower complementary, even as low as 50 % or more, may work. However, 100 % complementary is preferred.

Single stranded DNA can be designed to bind to genomic DNA in a sequence specific manner. Triplex Forming Oligonucleotides (TFO) are comprised of pyrimidine-rich oligonucleotides which bind DNA helices through Hoogsteen Base-pairing. The resulting triple helix composed of the DNA sense, DNA antisense, and TFO disrupts RNA synthesis by RNA polymerase.

Double-stranded RNAs can suppress expression of homologous genes through an evolutionarily conserved process named RNA interference (RNAi). One mechanism underlying silencing is the degradation of target mRNAs by an RNP (RiboNucleoProtein) complex, which contains short interfering RNAs (siRNAs) as guides to substrate selection.

The term "siRNA" refers to a short (typically less than 30 nucleotides long) double stranded RNA molecule. Typically, the siRNA modulates the expression of a gene to which the siRNA is targeted. Selection of a suitable small interfering RNA (siRNA) molecule requires knowledge of the nucleotide sequence of the target mRNA, or gene from which the mRNA is transcribed. The siRNA molecules of the invention are typically between 10-30 nucleotides in length, and preferably between 18-23 nucleotides in length. The siRNA molecules may comprise a sequence identical or at least 90% identical to any portion of the target gene whose expression is to be modulated including coding and non-coding sequences.

To provide a siRNA that can specifically suppress the expression of Epac, the following guidelines are used according to Elbashir et al., 2002 : 1) Selection of the target region from the open reading frame (ORF) of the cDNA sequence preferably 50 to 100

nucleotides downstream of the start codon. 2) Determination of a 21 nucleotide sequence in the target mRNA that begins with an AA dinucleotide (Elbashir et al., 2001). Thus sequences are 5'-AA(N19)UU, where N is any nucleotide. Sequences must contain approximately 50% G/C. 3) Blast-search (www.ncbi.nih.go/BLAST) the selected siRNA sequences against EST libraries or mRNA sequences of the respective organism to ensure that only a single gene is targeted. Any target sequences with more than 16 to 17 contiguous base pairs of homology to other coding sequences must be eliminated. 4) Synthesis of several siRNA sequences are advisable to control for the specificity of the knock-down experiments .

A short hairpin RNA is a simple strain RNA, characterized in that the two ends of said RNA are complementary and can hybridize together, thus forming an artificial double strand RNA with a loop between the two ends.

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it. A major advantage of this approach is that, because the ribozymes are engineered to be sequence-specific, only mRNAs with sequences complementary to the construct containing the ribozyme are inactivated. There are two basic types of ribozymes namely, tetrahymena-type and "hammerhead"-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences from about 3 to 18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating a specific mRNA species.

In accordance with the present invention, ribozyme, siRNAs, single strand DNA, or antisense oligonucleotides may be produced by expression of DNA sequences cloned into plasmid or retroviral vectors. Using standard methodology known to those skilled in the art, it is possible to maintain the ribozyme, siRNA or antisense oligonucleotides in any convenient cloning vector.

Various genetic regulatory control elements may be incorporated into ribozyme, siRNA, single stranded DNA, or antisense oligonucleotides expression vectors to facilitate propagation in cardiac cells. For instance, the cardiac specific expression of the siRNA may be driven by a cardiac specific promoter such as the α-Myosin Heavy Chain promoter.

A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

A "replicon" is any genetic element, for example, a plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e. g. , ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

According to another embodiment, the present invention relates to a method for treating a pathology selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis, in a patient, comprising administering to said patient a therapeutically effective amount of at least one Epac antagonist.

The invention relates to the method as defined above, wherein the antagonist is selected from the list comprising Epac activation inhibitors, Epac activity inhibitors, Epac intracellular localization disruption agents, and Epac expression inhibitors.

The invention also relates to a method as defined above, wherein the antagonist is an Epac activation inhibitor selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against Epac cAMP binding sites,
- cAMP analogues, such as cAMP derivatives or 8-(4-chlorophenylthio)-2'-O-methyladenosine-3'-5-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) derivatives,
- Brefeldin A or derivatives thereof.

The daily dosage of said antagonists may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 10 mg/kg of body weight per day. It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will

depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability, and length of action of that compound, the age, the body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the antagonist of Epac, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

In the pharmaceutical compositions of the present invention, the active principle is generally formulated as dosage units containing from 0.5 to 1000 mg, preferably from 1 to 500 mg, more preferably from 2 to 200 mg of said active principle per dosage unit for daily administrations.

The invention further relates to a method as defined above, wherein the antagonist is an Epac activity inhibitor, in particular an inhibitor of the Guanine nucleotide Exchange Factor (GEF) domain of Epac, or an inhibitor of the Ras Exchange Motif (REM) domain of Epac, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against the GEF domain or the REM domain of Epac,
- Brefeldin A or derivatives thereof.

The invention also relates to a method as defined above, wherein the antagonist is an Epac intracellular localization disruption agent, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against an Epac cellular localization domain, such as the Dishevelled Egl-10 Pleckstrin (DEP) domain,
- Brefeldin A or derivatives thereof.

The invention also relates to a method as defined above, wherein the antagonist is an Epac expression inhibitor selected from the list comprising:

- an antisense nucleic acid directed against Epac mRNAs,
- a double stranded RNA, a siRNA or a shRNA comprising Epac nucleic acid sequences,
- a ribozyme directed against Epac mRNAs.

According to another embodiment, the present invention relates to a pharmaceutical composition comprising as active substance an Epac expression inhibitor selected from the list comprising:

- an antisense nucleic acid directed against Epac mRNAs,
- a double stranded RNA, a siRNA or a shRNA comprising Epac nucleic acid sequences,
- a ribozyme directed against Epac mRNAs.

in association with a pharmaceutically acceptable carrier.

Ribozyme, siRNA, or antisense oligonucleotides encoding vectors and constructs as described herein are generally administered to a subject as a pharmaceutical preparation. As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. Preferably a subject according to the invention is a human

The pharmaceutical preparation comprising the ribozyme, siRNA or antisense oligonucleotides molecules or vectors are conveniently formulated for administration with an acceptable medium such as water, buffered saline, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), dimethyl sulfoxide (DMSO), oils, detergents, suspending agents or suitable mixtures thereof. The concentration of the ribozyme, siRNA or antisense oligonucelotides molecules or vectors in the chosen medium may depend on the hydrophobic or hydrophilic nature of the medium, as well as the length and other properties of the ribozyme siRNA or antisense oligonucelotides molecules or vectors. Solubility limits may be easily determined by one skilled in the art.

Ribozyme, siRNA or antisense oligonucleotides molecules and vectors encoding the same may be administered parenterally by intravenous injection into the blood stream, by subcutaneous, intramuscular, or intraperitoneal injection, or any other method known in the art. Pharmaceutical preparations for parenteral injection are commonly known in the art. If parenteral injection is selected as a method for administering the molecules or vectors, steps must be taken to ensure that sufficient amounts of the molecules or vectors reach their target cells to exert a biological effect. Several techniques have been used to increase the stability, cellular uptake and biodistribution of oligonucleotides. Ribozyme, siRNA or antisense oligonucleotides molecules of the present invention may be encapsulated in a lipophilic, targeted carrier, such as a liposome.

A pharmaceutical preparation of the invention may be formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to a physically discrete unit of the pharmaceutical preparation appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to

produce the desired effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate dosage unit are well known to those skilled in the art. Dosage units may be proportionately increased or decreased based on the weight of the patient. Appropriate concentrations for alleviation of a particular pathological condition may be determined by dosage concentration curve calculations, as known in the art.

In accordance with the present invention, the appropriate dosage unit for the administration of ribozyme-siRNA or antisense oligonucleotides molecules may be determined by evaluating the toxicity of the ribozyme, siRNA or antisense oligonucelotides molecules in animal models. Various concentrations of said molecules in pharmaceutical preparations may be administered to mice and the minimal and maximal dosages may be determined based on comparing obtaining desired results as opposed to side effects as a result of the treatment.

The pharmaceutical preparation comprising the molecules may be administered at appropriate intervals, for example, twice a day until the pathological symptoms are reduced or alleviated, after which the dosage may be reduced to a maintenance level.

The pharmaceutical composition is advantageously substantially pure. The term "substantially pure" refers to a preparation comprising at least 50-60% by weight of a given material (e.g. nucleic acid, oligonucleotide). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-95% by weight of the given compound. Purity is measured by methods appropriate for the given compound (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

The invention relates in particular to pharmaceutical compositions comprising as active substance an Epac activation inhibitor, an Epac activity inhibitor, or an Epac intracellular localization disruption agent, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against Epac cAMP binding sites,
- antibodies, fragments thereof, or aptamers, directed against the GEF domain or the REM domain of Epac,
- antibodies, fragments thereof, or aptamers, directed against an Epac cellular localization domain, such as the DEP domain,

in association with a pharmaceutically acceptable carrier.

The invention also relates to the use of Epac for screening compounds intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis.

These compounds are particularly chosen among Epac activation inhibitors, Epac activity inhibitors, Epac intracellular localization disruption agents, and Epac expression inhibitors.

The invention further relates to a method for screening compounds intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis, comprising the steps of:

- contacting Epac with a compound to screen in the presence of cAMP or an Epac activating cAMP analogue,
- assaying Epac activation,
- selecting compounds which inhibit Epac activation, in particular compounds which inhibit at least 30% of Epac activation, as compared to Epac activation in the absence of said compounds.

The cAMP-dependent activation of Rap1 can be assessed to determinate the percentage of Epac activation.

The invention also relates to the method as defined above, wherein Epac is also contacted with an effector protein liable to be activated upon Epac activation, such as Rap1, Rap2, Rac or Ras, and Epac activation is assessed by measuring the activity of said effector protein.

The screening method thus involves the steps of

- a) incubating Epac with i) at least one effector of Epac, chosen among, but not limited to, Rap1, Rap2, Rac or Ras, ii) cAMP and ii) said compound to be tested,
- b) assessing the activation of said effector by Epac,

wherein a lower activation, in comparison with the activation provided in absence of said compound to be tested, indicates that said compound is an antagonist of Epac. The assay of effector activation could be performed as hereunder described in Example 1 "Rac activation assay", ie wherein said effector is coupled in frame with GST.

In a particular embodiment, the screening method comprises testing *in vitro* the effect of the compounds to inhibit Epac-induced cAMP-dependent Rap1 or Rap2 activation. Rap1 is a well known effector of Epac. To do that, full-length Epac is incubated with Rap1 or Rap2 loaded with fluorescently labelled 2', 3'-bis(O)-N-methylantharanoloyl-guanosinediphosphate (mGDP) and the release of mGDP is detected in real time by a fluorescence spectrometer as previously described (Van den Berghe et al., 1997). The inhibition effect of the compounds on

Epac-induced cAMP-dependent Ras or Rac activation can be assessed using GST pull down assay as previously described (Maillet et al., 2003).

According to another embodiment, the present invention relates to a method for screening compounds intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis comprising the steps of:

- contacting cardiomyocytes having increased Epac activity as compared to normal cardiomyocytes with compounds to screen,
- assessing the hypertrophic properties of said cardiomyocytes having increased Epac activity,
- selecting compounds which inhibit the hypertrophic properties of said cardiomyocytes having increased Epac activity, as compared to the hypertrophic properties of cardiomyocytes having increased Epac activity not treated by said compounds.

In a further embodiment, the screening method involves the step of:

a) providing cardiomyocytes that express an activated form of Epac

b) exposing said cardiomyocyte to one of the compounds to be tested

c) assessing the effects of said compound on the hypetrophic properties of said cardiomyocytes, the hypertrophic properties being determined by measuring cell size and / or the expression of mRNA coding for hypertrophic gene markers, such as the natriuretic factor.

The cardiomyocytes that have an increased Epac activity as compared to normal cardiomyocytes express for example a wild type form of Epac or a constitutive activated form of Epac lacking its cAMP binding domain.

The inhibition of the hypertrophic properties indicates that said compound is an antagonist of Epac.

Administration of vectors producing ribozyme, siRNA or antisense oligonucleotides may be administered to cardiac cells or cardiac cell lines by any method such as, without limitation, transfection, electroporation, lipofection, and transduction.

In a further embodiment, the screening method can result in the combination of the two above described methods.

The invention further relates to the method as defined above, wherein the compounds to screen are:

- cAMP analogues, such as cAMP derivatives or 8-(4-chlorophenylthio)-2'-O-methyladenosine-3'-5-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) derivatives, or
- Brefeldin A derivatives.

A further object of the invention resides in a method for screening a compound, and especially a cAMP analog, that affects the activation of Epac.

According to another embodiment, the present invention relates to a non-human transgenic mammal for use as a model of cardiac hypertrophy, wherein Epac activity is increased with respect the corresponding wild type non-human mammal.

In this non human model of cardiac hypertrophy, cardiac hypertrophy has been induced by the specific expression of a positive dominant form of Epac, and especially Epac1 in cardiomycoytes.

The invention relates in particular to a non-human transgenic mammal for use as a model of cardiac hypertrophy as defined above, wherein Epac is over-expressed with respect the corresponding wild type non-human mammal, in particular said non-human transgenic mammal comprises Epac coding sequences under the control of cardiac-specific promoters having a stronger transcription activity than Epac natural promoter, such as the promoter of the α-Myosin Heavy Chain.

The positive dominant form of Epac is under the control of a cardiac specific promoter such as α-Myosin Heavy Chain.

The invention further relates to a non-human transgenic mammal for use as a model of cardiac hypertrophy as defined above, wherein said non-human transgenic mammal comprises nucleic sequences encoding a constitutively activated form of Epac lacking the activating cAMP binding domain.

In a further embodiment the positive dominant form of Epac1 results in Epac-ΔcAMP which contains a deletion of the first 322 amino acids of Epac1 (de Rooij et al., 1998). Epac-ΔcAMP lacks the cAMP-binding domain and therefore behaves as a constitutive activated form of Epac and cannot be regulated by cAMP (de Rooij et al., 1998).

The invention also relates to a non-human transgenic mammal for use as a model of cardiac hypertrophy as defined above, wherein said mammal is a mouse.

Therapeutic methods

The compositions containing the Epac antagonist(s) can be administered for prophylactic and/or therapeutic treatments. The active ingredient in the pharmaceutical composition generally is present in an "effective amount". By an "effective amount" of a pharmaceutical composition is meant a sufficient, but nontoxic amount of the agent to provide the desired effect. The term refers to an amount sufficient to treat a subject (e.g., a mammal, particularly a human). Thus, the term "therapeutic amount" refers to an amount sufficient to

remedy a disease state or symptoms, by preventing, hindering, retarding or reversing the progression of cardiac hypertrophy or any other undesirable symptoms whatsoever. The term "prophylactically effective" amount refers to an amount given to a subject that does not yet present the symptoms of cardiac hypertrophy, and thus is an amount effective to prevent, hinder or retard the onset of cardiac hypertrophy.

The symptoms of cardiac hypertrophy include shortness of breath on exertion, dizziness, fainting and angina pectoris. (Angina is chest pain or discomfort caused by reduced blood supply to the heart muscle.) Some people have cardiac arrhythmias. These are abnormal heart rhythms that in some cases can lead to sudden death. The obstruction to blood flow from the left ventricle increases the ventricle's work, and a heart murmur may be heard. In the majority of patients with hypertrophic cardiomyopathy, the physical examination is unremarkable and the abnormalities may be subtle. Most patients have forceful or jerky pulse and a forceful heart beat, which can be felt on the left side of the chest. Both of these reflect the thickened, strongly contracting heart. However the most obvious abnormality on physical examination is a heart murmur, which is present in 30 - 40% of patients. To diagnose hypertrophic cardiomyopathy, the electrocardiogram (ECG) is usually used. ECG usually shows an abnormal electrical signal due to muscle thickening and disorganization of the muscle structure. In a minority of patients (approximately 10%) the ECG may be normal or show only minor changes. As MRI (Magnetic Resonance Imaging) can provide tomographic high resolution pictures of the heart, it has recently become an important new test well suited for the assessment of the size and extent of left ventricular hypertrophy in cardiac hypertrophy. In fact, recent studies have shown that a cardiac MRI may be better than an echocardiogram to reliably detect hypertrophy in areas such as the left ventricular anterolateral wall and apex. As a result, in some patients an echocardiogram may not be sufficient to confidently exclude a diagnosis of cardiac hypertrophy and in that situation a cardiac MRI may be recommended.

In therapeutic applications, compositions are administered to a patient already suffering from a disease, as just described, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An appropriate dosage of the pharmaceutical composition is readily determined according to any one of several well-established protocols. For example, animal studies (e.g., mice, rats) are commonly used to determine the maximal tolerable dose of the bioactive agent per kilogram of weight. In general, at least one of the animal species tested is mammalian. The results from the animal studies can be extrapolated to determine doses for use in other species, such as humans for

example. What constitutes an effective dose also depends on the nature and severity of the disease or condition, and on the general state of the patient's health.

In prophylactic applications, compositions containing at least one Epac antagonists are administered to a patient susceptible to or otherwise at risk of a cardiac hypertrophy. Such an amount is defined to be a "prophylactically effective" amount or dose. In this use, the precise amounts again depend on the patient's state of health and weight.

The following examples are provided to illustrate certain aspects of the methods and compositions that are provided but should not be construed to limit the scope of the claimed invention.

DESCRIPTION OF THE FIGURES

Figure 1A, Figure 1B and Figure 1C

Epac activates the small G protein Rac in primary rat ventricular cardiomyocytes and HL-1 atria cells.

Figure 1A: Primary rat ventricular cardiomyocytes were infected with either Ad.GFP as a control or with Ad.EpacWT or Ad.Epac-ΔcAMP as described in Methods. Two days after infection, cells were treated or not for 10 min with the selective activator of Epac, 8-CPT (1 μM). Amounts of Rac-GTP were determined by pull down experiments. A control for total Rac expression (total lysates) is shown. The upper panel shows a typical immunoblot. Expression of recombinant proteins was determined by Western blot using an anti-HA antibody. The lower panel shows means ± S.E.M. of 3 independent experiments. Results are expressed as fold activation of control cells infected with Ad.GFP. *$p<0.05$, **$p<0.01$ compared with control.

Figure 1B: HL-1 atrial cells were treated with 8-CPT (100 μM), Forskolin (FSK) (100 μM), or 8-Br-cAMP (10 μM) for 10 min. The upper panel shows a representative immunoblot. In lower panel, Rac-GTP is expressed as fold activation of the control (CT) cells (means ± S.E.M; n=4).

Figure 1C : Effect of 8-CPT (10 μM) at different time of incubation on the amount of Rac-GTP. Rac activation was determined as above. Control for total lysates (Total Rac) is shown.

Figure 2A, Figure 2B, Figure 2C and Figure 2D

Epac stimulates a hypertrophic pattern of gene expression.

Figures 2A, 2C and 2D: Neonatal cardiomycoytes were transfected with ANF-Luc (Figure 2A), SkM-α-actin-Luc (Figure 2C) or c-fos-SRE-Luc (Figure 2D) and EpacWT, RacG12V or the empty vector (mock) as control and treated or not with 8-CPT (1 μM). Two days after transfection, cells were assayed for luciferase activity. Results are expressed as percentage activation of control. Results are means ± S.E.M. for 3 independent experiments performed in triplicates.

Figure 2B: Epac induces expression of ANF mRNA. Cardiomyocytes were infected with Ad.EpacWT, Ad.RacG12V, or Ad.GFF (control) and stimulated or not with 8-CPT (1 μM) for 2 days. ANF mRNA expression was determined by quantitative PCR as described in Methods. Values are expressed relative to the ANF/GCB ratio and results were normalized to control for each experiment. Results are presented as the mean ± S.E.M. of 3 independent experiments performed in duplicates. *p<0.05, **p<0.01, ***p<0.001 compared with control.

Figure 3A, Figure 3B, Figure 3C, Figure 3D and Figure 3E

Epac induces cardiomyocytes hypertrophy in neonatal (Figures 3A, 3B and 3C) and adult (Figure 3D and 3E) rat primary cardiomyocytes.

Figure 3A: Fluorescent microscopic analyses of the effects of Epac on sarcomeric organization. Morphology of representative myocytes 48 h after infection with Ad.GFP as a control, or Ad.EpacWT is shown. The Epac selective activator, 8-CPT, was used at 1 μM for 2 days in cells infected with Ad.GFP. For a positive control, cells were infected with Ad.GFP and treated with PE (1 μM) for 2 days. Actin filaments were visualized by using Rhodamin-conjugated phalloidin.

Figure 3B: Photographic images of cells infected for 2 days with Ad.GFP or Ad.EpacWT and treated or not with either 8-CPT (1 μM) or PE (1 μM) were digitised. The areas (μm^2) of 30 to 50 individualized cells per condition from 2 to 3 independent experiments were determined by computer-assisted planimetry. Values show the means ± S.E.M.

Figure 3C: [^{3}H]-leucine incorporation. Cardiomyocytes were treated as above and total radioactivity of incorporated [^{3}H]-leucine into proteins was determined by scintillation counting. The figure shows the mean ± S.E.M. of data for 3 experiments performed in duplicate. *p<0.05, **p<0.01, ***p<0.001 compared with control Ad.GFP.

Figure 3D: α-actinin staining of cardiac myocytes infected with the Ad.GFP or the Ad.Epac^{WT} and GFP, or Ad.Epac^{dcAMP}, or Ad.Rap^{Q63E}, treated or not with an agonist of Epac, 8-CPT. Morphology of representative myocytes 48 h after infection with Ad.GFP as a control, Ad.Epac^{WT}/GFP or Ad.Rap^{Q63E} is shown. The Epac selective activator, 8-CPT was used at 1

μM for 2 days. α-actinin was visualized by immunocytochemistry as described in Methods. Pictures show from the upper to the lower panel: α-actinin staining (upper panel), GFP expression (middle), and merge from the two previous pictures (lower panel).

Figure 3E: Photographic images of rat adult cardiac myocytes infected for 2 days with the Ad.GFP, or Ad.Epac^{WT}, or Ad.Epac^{dcAMP} or Ad.Rap^{Q63E} and treated or not with 8-CPT (1 μM) and were digitized. The surface, the length, and the width of around 100 individualized cells per condition from 5 to 6 independent experiments were determined by computer-assisted planimetry. Values show the means ± S.E.M.

Figure 4A, Figure 4B, Figure 4C, Figure 4D and Figure 4E

Epac induces intracellular Ca2+ transients and Ca2+ activates Rac.

In figures 4A, 4B, 4C and4D, neonatal cardiomyocytes at day 1 or 2 after plating were loaded with the Ca2+ indicator Fluo3-AM and perfused with a control external ringer solution.

Figure 4A: Effect of 10 μM 8-pCPT-2'-O-Me-cAMP (8-CPT) on spontaneous spiking activity at 1.8 mM external [Ca^{2+}].

Figure 4B: Effect of 10 μM 8-CPT in the presence of 20 mM external Cs^{+}.

Figure 4C: Effect of 100 μM 8-CPT in the absence of external Ca^{2+}.

Figure 4D: Effect of 100 μM 8-CPT in the absence of external Ca^{2+} and presence of the PKA blocker H89 (1 μM).

Figure 4E: Effect of ionomycin on Rac activation. Primary rat ventricular cardiomyocytes were treated at 2 days *in vitro* with ionomycin (1 μM) for different times of incubation or PE (1 μM) for 15 min as a positive control. The amount of Rac-GTP was determined by pull down experiments followed by Western bloting using an anti-Rac antibody.

Figure 5A, Figure 5B and Figure 5C

Activation of NFAT by Epac.

Figures 5A and Figure 5B: Effect of Epac on NFAT transcriptional activity. Cardiomyocytes infected with Ad.GFP (control), Ad.EpacWT, or Ad.VIVIT were transfected with NFAT-Luc and treated or not with CsA (0,5 μM) for 48 h. Luc activity was assayed as described in Methods.

Figure 5C: Epac increases MCIP1 expression. Cardiac myocytes infected with Ad.GFP (control) or Ad.EpacWT were treated or not with 8-CPT (1 μM) for 2 days. The ratio of MCIP1/GCB mRNA was determined by quantitative PCR. Values are expressed relative to the MCIP1/GCB ratio.

Results were normalized to control for each experiment, and were expressed as means ± S.E.M of at least 3 independent experiments performed in triplicate (Figures 5A and 5B) or duplicate (Figure 5C).

Figure 6A and Figure 6B

Ad.VIVIT inhibits Epac-induced cardiomyocyte hypertrophy

Figure 6A: Fluorescent microscopic analyses of the effects of Epac on sarcomeric organization. Morphology of representative myocytes 48 h after infection with Ad.GFP as a control, Ad.VIVIT, Ad.EpacWT, or Ad.EpacWT and Ad.VIVIT is shown.

Figure 6B: Photographic images of cardiac myocytes infected as above were digitised. Areas (μm^2) of around 50 individualized cells per condition from 3 independent experiments were determined by computer-assisted planimetry. Values show the means ± S.E.M. *$p<0.05$, **$p<0.01$ and *** $p<0.001$ compared with control or versus indicated values.

Figure 7A, Figure 7B and Figure 7C

Epac activates CaMKII signaling pathway.

Figures 7A: Epac regulates MEF2 transcriptional activity. Cardiomyocytes infected with Ad.GFP (control), Ad.EpacWT, or Ad.EpacΔcAMP were transfected with MEF2-Luc and treated or not with CsA (0,5 μM) for 48 h. Luc activity was assayed as described in Methods.

Figure 7B: Epac regulates MEF2 transcriptional activity. Cardiomyocytes infected with Ad.GFP (control), Ad.EpacWT, or Ad.EpacΔcAMP were transfected with MEF2-Luc and treated or not with KN-93 (1 μM) for 48 h. Luc activity was assayed as described in Methods.

Figure 7C: KN-93 inhibits Epac-induced cardiomyocyte hypertrophy. Cardiomyocytes infected with Ad.GFP (control), or Ad.EpacΔcAMP and treated or not with KN-93 (1 μM) for 2 days were stained with phalloidin. Photographic images were then taken and digitised. Areas (μm^2) of around 50 individualized cells per condition from 3 independent experiments were determined by computer-assisted planimetry. Results were normalized to control for each experiment, and were expressed as means ± S.E.M of at least 3 separate experiments performed in triplicate.

Figure 8A, Figure 8B and Figure 8C

Involvement of Rac in Epac-induced NFAT dependent cardiomyocyte hypertrophy.

In figures 8A, 8B and 8C, cardiomyocytes infected with Ad.GFP (control), Ad.RacS17N, Ad.EpacWT, or Ad.EpacWT and Ad.RacS17N were transfected with NFAT-Luc, MEF2-Luc

or ANF-Luc. Two days after, Luc activity was determined. Luc activity was normalized to control for each experiment, and were expressed as means ± S.E.M of at least 3 separate experiments performed in triplicate

In figure 8B, lower panel, the expression of the infected constructs was monitored using antibodies directed against HA and c-Myc.

Figure 9A and Figure 9B

Ad.RacS17N reverses Epac-induced cardiomyocyte hypertrophy.

Figure 9A: Photographic images of cells infected for 2 days with Ad.GFP (control), Ad.EpacWT, Ad.RacS17N, or Ad.EpacWT and Ad.RacS17N were digitised.

Figure 9B: The areas (μm^2) of 50 individualized cells per condition from 3 independent experiments were determined by computer-assisted planimetry. Values show the means ± S.E.M. *$p<0.05$, **$p<0.01$ and *** $p<0.001$ versus control cells or indicated values.

Figure 10

Possible hypertrophic signaling pathways controlled by Epac.

Stimulation of Gs-coupled receptor activates the adenylyl cyclase (AC) which increases intracellular cAMP levels. Subsequent activation of Epac induces a rise in [Ca2+]i which increases Rac activation. The small G protein Rac can both induce cytoskeletal reorganization and activation of the calcineurin / NFAT signaling pathway. Epac also regulates MEF2 transcriptional activity via CaMKII. Epac signaling pathway induces hypertrophic gene expression and cardiomyocyte growth.

Figure11

Activation of Epac1 increases protein synthesis in adult rat primary cardiomyocyte. Cardiomyocytes were infected with the Ad.GFP (control), or the $Ad.Epac^{WT}$ and GFP, or $Ad.Rap^{Q63E}$, treated or not with an agonist of Epac, 8-CPT, and total radioactivity of incorporated [3H]-leucine into proteins was determined by scintillation counting. The Epac selective activator, 8-CPT was used at 1 μM for 2 days. As a positive control, cells were treated with the hypertrophic stimulus, phenylephrine for 2 days (1 μM). The figure shows the mean ± S.E.M. of data for 3 experiments performed in duplicate. *$p<0.05$, **$p<0.01$, compared with control Ad.GFP.

Figure 12

Epac1 is increased in patients with heart failure (HF, n=22).

Change in gene expression was analyzed by quantitative RT-PCR. Histograms show the relative mRNA abundance of human Epac1. Data are expressed as fold over control patients (n= 11) normalized to the housekeeping gene GCB. Results are mean values ± SEM.

Figure 13

Schematic representation of a bicistronic adenovirus (Ad.) bearing either $Epac^{WT}$ (wild type) or $Epac^{\Delta cAMP}$ (constitutive activated form of Epac1) under the control of a cytomegalovirus (CMV) promoter, and green fluorescent protein (GFP) under internal ribosomal entry site (IRES) control.

Figure 14

Schematic representation of the α-MHC-HA-EpacΔAMP.

The cDNA coding for the human form of Epac1 lacking its first 322 amino acids (EpacΔcAMP) and containing a HA epitope (HA) in its N terminus was fused upstream the α-Myosin Heavy Chain cardiac specific promoter (α-MHC).

EXAMPLES :

EXAMPLE 1: The cAMP-binding protein Epac induces cardiomyocyte hypertrophy

Materials and Methods

Materials

All media, sera and antibiotics used in the cell culture were purchased from Invitrogen (Cergy Pontoise, France). 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3'-5'cyclic monophosphate (8-pCPT-2'-O-Me-cAMP) was from Biolog Life Science Institute (Bremen, Germany). Forskolin, 8-Bromo-cAMP, Phenylephrine and H89 were obtained from Calbiochem (France Biochem, Meudon, France).

Cell culture

HL-1 atrial cardiomyocytes, a gift from Dr. Claycomb (Louisiana State University, New Orleans, LA, U.S.A.) were plated onto fibronectin-gelatin-coated plates or coverslips and cultured in Claycomb medium supplemented with 10% fetal bovine serum, 100 units/ml

penicillin, 100 µg/ml streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine as described (Claycomb et al, 1998).

Neonatal rat ventricular myocytes were isolated according to the protocol described by Wollert and colleagues (Wollert et al. 1996) and modified as follows: ventricular cells from one day old wistar rats were individualized by digestion with collagenase A (Roche Diagnostics Corporation, Germany) and pancreatin. The cell suspension was purified by centrifugation through a discontinuous Percoll gradient (Sigma Aldrich, L'Isle d'Abeau Chesmes, France). Cardiomyocytes were plated in gelatin-coated culture dishes in Dubelcco's modified Eagle's medium (DMEM)/medium 199 (4/1) supplemented with 10 % horse serum (v/v), 5% newborn calf serum (v/v), glutamine and antibiotics. The day after, cardiomyocytes were switched to the maintenance medium composed of DMEM/medium 199 supplemented only with glutamine and antibiotics.

Adult rat ventricular myocytes were isolated from male Wistar rats (160–180 g). The rats were subjected to anesthesia by intraperitoneal injection of pentothal (0.1 mg/g), and hearts were excised rapidly. Individual ventricular myocytes were obtained by retrograde perfusion of the heart as previously described (Verde et al., 1999). Freshly isolated cells were suspended in minimal essential medium (MEM: M 4780; Sigma) containing 1.2 mM Ca^{2+}, 2.5% fetal bovine serum (FBS, Invitrogen, Cergy-Pontoise, France), 1% penicillin-streptomycin and 2% HEPES (pH 7.6) and plated on laminin-coated culture dishes (10 µg/ml laminin, 2 h) at a density of 10^5 cells per dish. The cells were left to adhere for 1 h in a 95% O_2, 5% CO_2 incubator at 37 °C before adenoviral infection.

Adenoviral Infection

Bicistronic adenoviruses (Ad5) bearing either EpacWT or EpacΔcAMP under the control of a cytomegalovirus (CMV) promoter, and green fluorescent protein (GFP) under internal ribosomal entry site (IRES) control were constructed and amplified at the Genethon Center of Evry (France) (**Figure 13**). Adenoviruses encoding VIVIT, a selective peptide inhibitor of calcineurin-mediated NFAT activation, and Rac were provided by Drs. J. Molkentin and T. Finkel, respectively. One day after plating, cardiomyocytes were incubated for 2 h with recombinant adenoviruses. After removal of the virus suspension, cells were replaced in maintenance medium for 2 days and then stimulated with the different drugs. Viruses were used at a multiplicity of infection (MOI) of 100.

Concerning the adenoviral infection of adult rat ventricular myocytes, the cells were left to adhere for 1 h in a 95% O_2, 5% CO_2 incubator at 37 °C, before the medium was

replaced by 200 µl of Fetal Bovine Serum (FBS)-free MEM containing the Epac1 wild type (WT)-encoding adenovirus (Ad.Epac^{WT}), the constitutive active form of Epac1 (Ad.EpacΔcAMP) adenovirus, the constitutive active form of Rap1 (Ad.Rap1^{Q63E}) or the green fluorescent protein-encoding adenovirus (Ad.GFP). Ad.Epac^{WT}, Ad. Rap1^{Q63E} or Ad.GFP were used at a multiplicity of infection (MOI) of 100 plaque-forming units (pfu) per cell, whereas Ad.EpacΔcAMP was generally used at 500 MOI (see results). After 2 h, the same volume of FBS-free medium without adenovirus was added, and the cells were placed for 2 days in an incubator (37°C, 5%CO2). The medium was changed the next morning for adenovirus- and FBS-free MEM.

Plasmid constructs and transfection

The plasmid constructs were generously provided by the following: a –3003 bp fragment of the rat ANF promoter fused to the luciferase reporter gene (ANF-Luc) by Dr. K. Knowlton, Luciferase reporter genes linked to promoters for skeletal muscle α-actin (SkM-α-actin-Luc) and serum response element-regulated c-fos (c-fos-SRE-Luc) by Dr. M. D. Schneider, Epac1 plasmid constructs by Dr. J. Bos. The luciferase reporter plasmid driven by four NFAT consensus binding sites (NFAT-Luc) or driven by the three MEF2 consensus binding sites (MEF2-Luc) was obtained from Stratagene and was kindly provided by Dr KC Wollert respectively. Transient transfection experiments were performed with Lipofectamine 2000 (Invitrogen Life Technologies, France) in optimem medium in the presence of 1 mg of the various plasmid constructs according to the manufacturer's instructions. Two days post-transfection, cells were lysed and assayed at 37°C for Luciferase activity using a Luciferase assay kit (promega corporation, Madison USA) in conjunction with a luminometer allowing automated luciferin solutions injection (Lumat LB 9507, EG & G Berthold).

[3H]-leucine incorporation

The assessment of protein synthesis was achieved by adding 1 mCi/ml of [3H]-leucine (Amersham Chemical Corp.) to each well for 24 h (neonatal cardiomyocytes) or 48 h (adult cardiomyocytes) in presence of adenoviruses and/or in stimulated conditions in the maintenance medium. Thereafter, the [3H]-leucine-containing medium was aspirated. Myocytes were washed three times with phosphate-buffered saline (PBS) and incubated with 5% trichloroacetic acid for 30 min at 4°C. The cell residues were rinsed in 70% and then 100% ethanol, solubilized in 0.33 M NaOH for 1 h and scrapped with a rubber policeman. Radioactivity was measured in a liquid scintillation counter (LS 6000 SC Beckman).

Actin staining and surface area

Cardiomyocytes were plated in Lab-Tek plastic chamber slides (Nunc), precoated with gelatin (0.2%) and incubated in the presence or absence of various agents. After 48 h, cells were rinsed three times with PBS containing 1% BSA and fixed by immersion in 4% paraformaldehyde during 30 min.

For actin cytoskeletal organization, cardiomyocytes were incubated with a 1/800 diluted solution of rhodamine phalloidine (Sigma Aldrich, L'isle d'Abeau Chesmes, France) for 45 min with gentle shaking.

Alternatively, cardiac actin organization was assessed by the following protocol: myocytes were incubated with 0.2% Triton X-100 for 5 min, followed by 0.5 mol / L NH_4Cl in PBS for 15 min. After a preincubation in 5% bovine serum albumin in PBS for 30 min, myocytes were incubated overnight with a mouse monoclonal antibody directed against cardiac sarcomeric α-actinin (cloneEA-53, 1/300, Sigma, France). The secondary antibody used was a goat anti-mouse IgG coupled to Alexa Fluor®594 (1/150, Molecular Probes).

Following washing with PBS, cells were finally mounted on glass coverslips in mowiol antifadent mounting medium (mowiol 8%, glycerol 8%, DABCO (di-aza-bicyclo-octane); France Biochem, Meudon, France). F-actin was analysed by confocal scanning laser microscope. Optical section series were obtained with a Plan Apochromat 63X objective (NA 1.4, oil immersion). Morphometric parameters were determined by computer-assisted planimetry (Perkin Elmer). Thirty to fifty individualized cells using the rhodamine phalloidine and 500 to 600 individualized cells using the cardiac sarcomeric α-actinin antibody were analyzed for each condition.

Rac activation assay

Rac pull-down experiments were performed using a GST fusion protein containing the Cdc42/Rac Interactive Binding Domain (CRIB) of PAK as previously described (Maillet et al, 2003). After stimulation, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5; 500 mM NaCl; 20 mM MgCl2; 0.5% deoxycholic acid; 0.1% SDS; 1% Triton X-100; 1 mM PMSF; 10 mg/ml leupeptin and aprotinin) and 2 mg of protein were incubated with GST-CRIB coupled to glutathione-sepharose beads (Amersham Biosciences) for 1h at 4°C. Beads were then washed three times in Rac washing buffer (50 mM Tris-HCl , pH 7.5; 150 mM NaCl, 20 mM MgCl2, 1% Triton X-100; 0.1 mM PMSF; 10 mg/ml leupeptin and aprotinin). Rac-GTP samples and total lysates were separated on SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech). Membranes

were hybridised with an anti Rac1 monoclonal antibody (Upstate Biotechnology) and proteins were revealed by enhanced chemiluminescence (ECL+; Amersham Pharmacia Biotech).

Intracellular Ca2+ measurements

Neonatal rat ventricular myocytes at day 1 to 2 after isolation were loaded with the Ca2+ indicator Fluo 3 AM (Molecular Probes, 10 µM, 30 min, 37°C) in serum-free maintenance medium, and then washed for additional 30 min in external ringer solution containing (mM) NaCl 121, KCl 5.4, Hepes 10, Glucose 5, Na-pyruvate 5, NaHCO3 4, Na2HPO4 0.8, MgCl2 1.8, CaCl2 1.8 . Experiments were carried out in this solution or in the ringer solution with 100 µM EGTA and without added Ca2+ and Mg2+ and. In order to block HCN channels, 20 mM CsCl was added to the initial Ca2+ solution, while KCl was omitted and NaCl was reduced to 107 mM to maintain osmolarity constant. Cells were mounted on the stage of an inverted Nikon Eclipse microscope and visualized using a 63X oil immersion fluorescence objective. The field was illuminated at 488 nm with a xenon lamp. Images at 535 nm were captured by a CCD camera (Sensicam QE, photoline) driven by Metafluor software. Experiments were performed at room temperature.

Reverse Transcription –Polymerase Chain Reaction (RT-PCR)

Left-ventricular samples obtained from human hearts were classified into two groups, control ("normal") donors and end-stage failing hearts. Total RNA was prepared from human tissue or from neonatal rat cardiomyocytes using the Trizol RNA purification system (Life Technologies Inc.). RNA was then treated with DNase I (Life Technologies Inc.) and 3 mg of total RNA were then hybridized with oligo(dT) primer and reverse transcribed using Superscript reverse transcriptase II (Life Technologies Inc.). Quantitative RT–PCR was conducted with a LightCycler system (Roche) using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche) and specific primer pair for human Epac1 (5'-GCTCTTTGAACCACACAGCA -3'; 5'-TGTCTTCTCGCAGGATGATG -3'), or ANF (5'-GGGCTCCTTCTCCATCACCAA-3'; 5'-CTTCATCGGTCTGCTCGCTCA-3') or MCIP1 (5'-AGCGAAAGTGAGACCAGGGC-3'; 5'-GGCAGGGGGAGAGATGAGAA-3'). PCR reactions were performed using the following cycle conditions: denaturation for 10 sec at 95°C, annealing for 5 s at 60°C and extension for 11 sec at 72°C. Dissociation curves were generated after each PCR run to ensure that a single specific product was amplified. Glucocerebrosidase (GCB) was measured as a reference gene using the primer pair 5'-GCACAACTTCAGCCTCCCAGA-3' and 5'-CTTCCCATTCACCGCTCCATT-3'.

Statistical Analysis

Results are expressed as means ± SEM. Differences between groups have been analyzed by one-way ANOVA followed by unpaired Student's t test. Differences were considered significant when * P<0.05, ** P< 0.01, *** P<0.001.

Results :

Epac activates the small G protein Rac in cardiomyocytes

The Inventors directly assayed Rac GTP-loading using a glutathione S-transferase (GST) fusion protein containing the Cdc42-Rac interactive binding domain (CRIB) of p21-activated kinase (PAK). Activation of endogenous Epac with a selective activator of this GEF, 8-pCPT-2'-O-Me-cAMP (8-CPT) (Enserink et al, 2002) increased Rac activation in rat cardiac myocytes (**Figure 1A**). Similarly, infection of cardiomyocytes with an adenovirus encoding Epac1WT (Ad.EpacWT) significantly enhanced Rac GTP-loading compared to control cells infected with GFP (**Figure 1A**). Rac activation was further increased when cells infected with Ad.EpacWT were treated with 8-CPT (1 μM) for 10 min (**Figure 1A**). As shown in **Figure 1A**, an adenovirus bearing a constitutive activated form of Epac1 (Ad.Epac-ΔcAMP) (de Rooij et al, 1998) significantly induced Rac activation. The fact that Ad.EpacWT had similar effects on Rac activity as observed for Ad.Epac-ΔcAMP could be explained by its activation with endogenous cAMP. The regulation of Rac activity by Epac was not restricted to ventricular cardiomyocytes since a 10 min exposure of HL-1 adult mouse atrial cells to 8-CPT (100 μM) caused a strong increase in the amount of Rac-GTP (**Figure 1B**). In this cell line, 8-CPT-induced Rac activation was detected upon 5 min treatment with this cAMP analogue (**Figure 1C**). Furthermore, the Inventors observed that a potent activator of adenylyl cyclase, forskolin (100 μM) and a cAMP analogue, 8-Br-cAMP (100 μM) mimicked the effect of 8-CPT on Rac activation (**Figure 1B**). Altogether, these results demonstrate that recombinant and native Epac induce the conversion of Rac into an activated state in atrial and ventricular cardiomyocytes.

Epac increases the expression of hypertrophy gene markers

As Rac has been found to be involved in cardiac myocyte hypertrophy (Pracyk et al, 1998; Sussman et al, 2000), the Inventors next tested the potential involvement of Epac in this process. Re-expression of embryonic genes and transient activation of immediate early genes are frequently used indexes of myocyte hypertrophy (Chien et al, 1991). The ability of Epac to stimulate gene expression was determined using luciferase (Luc) constructs under the control of promoters for atrial natriuretic factor (ANF), skeletal muscle (SkM) α-actin and the

c-fos-responsive element (c-fos-SRE). **Figure 2A** shows a three fold activation of the ANF-Luc reporter gene in neonatal cardiomyocytes stimulated with 8-CPT (1 μM) compared to control cells. Transient transfection of Epac1WT as well as 8-CPT (1 μM) increased the basal level of ANF-Luc activity (**Figure 2A**). A constitutive activated form of Rac (RacG12V) mimicked the effect of Epac on ANF-Luc activity (**Figure 2A**). Accordingly, endogenous expression of ANF mRNA was significantly increased in ventricular cardiomyocytes infected with Ad.EpacWT and stimulated or not with 8-CPT (1 μM), as compared to cell infected with control Ad.GFP (**Figure 2B**). Similar results were obtained with an adenovirus expressing Ad.RacG12V (**Figure 2B**). In addition, when cotransfection experiments were performed with SkM-α-actin-Luc or c-fos-SRE-Luc, it was found that EpacWT, Epac-ΔcAMP, or RacG12V significantly increased Luc activity compared to control cells (**Figure 2C and 2D**).

Epac increases cardiomyocyte size and sarcomeric organization

Further studies were undertaken to determine the effects of Epac on other features of the hypertrophic program such as cell size and sarcomeric organization. Cytoskeletal organization was analysed by phalloidin staining. Cardiomyocyte treatment with Ad.GFP and 8-CPT (1 mM) as well as infection of cardiomyocytes with Ad.EpacWT induced an apparent increase of the F-actin meshwork and an heavily striated appearance, reflecting the organization of this F-actin cytoskeleton into sarcomeric structures, as compared to cardiomyocytes infected with Ad.GFP alone (**Figure 3A**). The effects of Epac on sarcomeric organization were comparable to Ad.Epac-ΔcAMP (data not shown) and PE (1 mM) (**Figure 3A**), a well known inducer of cardiac hypertrophy. To further characterize the ability of Epac to induce morphological hypertrophy, the Inventors measured cell surface area. Activation of endogenous Epac with 8-CPT (1 μM) produced a two fold increase in cell surface area when compared to cardiac myocytes infected with control Ad.GFP (**Figure 3B**). Identical results were obtained when cardiomyocytes were infected with Ad.EpacWT (**Figure 3B**), Ad.Epac-ΔcAMP (data not shown), or Ad.GFP and treated with PE (1 μM) (**Figure 3B**). The effect of Ad.EpacWT on cell surface area was not further increased in the presence of 8-CPT (1 μM) suggesting that intracellular cAMP was sufficient to activate recombinant Epac to induce its maximal effect on protein synthesis (**Figure 3B**).

Similar results were obtained with adult rat primary cardiomyocytes. The actin organization was assessed using an antibody directed against cardiac sarcomeric α-actinin and then cell surface area was measured. Figures 3D and 3E show that cells infected with Ad.EpacWT in the presence of 8-CPT (1 μM), Ad.Epac-ΔcAMP or a constitutive activated

form of Rap1, Rap^{Q63E} significantly increased cell size as compared to control cells infected with GFP.

Finally, the effect of Epac on protein synthesis was analyzed by measurement of [3H]-leucine incorporation into cardiac myocytes. Consistent with the ability of Ad.EpacWT to increase cell surface area, expression of this cAMP-GEF resulted in an increase in [3H]-leucine uptake into cardiomyocytes (**Figure 3C**). Similarly, cell treatment with the Epac-selective cAMP analogue, 8-CPT (1 μM) or the gold standard, PE (1 μM) resulted in an approximate two fold increase in protein synthesis (**Figure 3C**). In addition, Ad.EpacWT in the presence of 8-CPT and Rap^{Q63E} significantly increased protein synthesis as compared to control cells infected with Ad.GFP in primary adult cardiac myocytes (**Figure 11**). Altogether, these results show that Epac activation confer all the features of the hypertrophic phenotype in primary ventricular cardiomyocytes.

Epac activation increases Ca2+ transients frequency

Alterations in intracellular Ca2+ handling progressively exacerbate a hypertrophic or cardiomyopathic phenotype, in part, through sustained activation of Ca2+-sensitive signal transduction pathways (Balke & Shorofsky, 1998). Given the involvement of Epac in cardiac hypertrophy, the Inventors examined whether its activation could affect intracellular Ca2+ concentration ([Ca2+]i) in neonatal myocytes (**Figure 4**). As seen in **Figure 4A**, at physiological external [Ca2+], these cells exhibited spontaneous Ca2+ transients with a low frequency (0.120 ± 0.015 Hz, n=20). Application of the Epac agonist 8-CPT (10 μM) triggered a dramatic increase in the frequency of these Ca2+ oscillations (0.51 ± 0.04 Hz, n=7) without changing the amplitude of the spikes. This effect was also observed at 100 nM 8-CPT (0.40 ± 0.05 Hz, n=13, data not shown). HCN channels underlie the pacemaker current If which is an important contributor of automaticity in neonatal ventricular cells (Er et al, 2003). If is directly regulated by cAMP and its activation by the cAMP analogue, 8-CPT would be expected to increase spontaneous diastolic depolarisation and rhythmic activity. To test this hypothesis, the Inventors analysed the effect of 8-CPT (10 μM) in the presence of 20 mM Cs+ in the external medium to block HCN channels. As seen in **Figure 4B**, external Cs+ failed to prevent the effect of 8-CPT on spontaneous Ca2+ transients, and rather amplified them. Indeed, in these conditions, spike frequency was increased from 0.09 ± 0.11 Hz to 1.25 ± 0.35 Hz by 8-CPT (n=8).

The Inventors next tested the dependence of the 8-CPT effect towards extracellular Ca2+. In the absence of this ion in the bath, spontaneous Ca2+ transients were virtually

abolished and 8-CPT (10 μM) had no effect (data not shown). However, increasing the concentration of the Epac agonist to 100 μM triggered the generation of Ca2+ spikes (0.004 ± 0.002 Hz in basal and 0.12 ± 0.02 Hz in the presence of 100 μM 8-CPT, n=4, **Figure 4C**). This effect was independent of PKA since an inhibitor of this kinase, H89 failed to block 8-CPT-induced increase in Ca2+ transients frequency (**Figure 4D**). In the presence of H89 (1 μM), 8-CPT (100 μM) increased the basal spike frequency from 0.012 ± 0.007 Hz to 0.23 ± 0.049 Hz (n=8). These data indicate that Epac activation produces bursts of Ca2+ transients in neonatal cardiac myocytes, in part by mobilizing an internal Ca2+ source.

As described above, Epac induced Rac activation. Therefore, the Inventors examined the dependence of Rac activation on Ca2+ signaling. Treatment of cardiac myocytes with the Ca2+ ionophore ionomycin (1 μM) increased Rac activation in a time dependent manner (**Figure 4E**). The effect of ionomycin on Rac activation was as potent as PE (1 μM) which was used as a positive control in the experiments (**Figure 4E**). From these results the Inventors conclude that elevation of intracellular [Ca2+]i is sufficient to activate Rac.

Epac activates the hypertrophic calcineurin/NFAT and MEF2 signaling pathways

To test whether Epac may activate the hypertrophic calcineurin NFAT signaling pathway, primary cardiomyocytes were transfected with a Luc reporter plasmid driven by four NFAT consensus binding sites (NFAT-Luc) and infected with Ad.EpacWT. As shown in **Figure 5A**, Ad.EpacWT significantly increased NFAT transcriptional activity as compared to control cells infected with Ad.GFP. In contrast, Ad.EpacWT had no effect on the promoter-less pGL3 basic vector (data not shown). Epac-induced NFAT transcriptional activity was blocked by a pharmacological inhibitor of calcineurin, cyclosporine A (CsA) (0.5μM) (**Figure 5A**). Consistent with this finding, an adenovirus bearing a selective peptide inhibitor of calcineurin named VIVIT (Ad.VIVIT) (Aramburu et al, 1999), blocked the stimulating effect of Ad.EpacWT on NFAT transcriptional activity (**Figure 5B**). In addition, the Inventors found that cardiac myocytes infected with Ad.EpacWT and treated or not with 8-CPT (1 μM) (**Figure 5C**), or Ad.Epac-DcAMP (data not shown) had an increased mRNA encoding the modulatory calcineurin-interacting protein 1 (MCIP1), a mediator of calcineurin signaling during cardiac hypertrophy (Yang et al, 2000). The Inventors also analysed the effect of Ad.VIVIT on the Epac-induced cytosketal reorganization into a sarcomeric structure by phalloidin staining. Co-infection with Ad.VIVIT and Ad.EpacWT reduced the enhancement of sarcomeric organization induced by Ad.EpacWT (**Figure 6A**). As expected, the ability of Ad.EpacWT to increase cell surface area was significantly decreased by Ad.VIVIT (**Figure**

6B). Altogether these data show that NFAT is a downstream component of Epac hypertrophic signaling pathway.

Next, the Inventors investigated whether MEF2 is also a target for Epac hypertrophic signaling. Infection of cardiomycytes with either Ad.EpacWT or Ad.Epac-DcAMP produced a strong increase in MEF2 transcriptional activity (**Figure 7A**). Because calcineurin has been shown to stimulate MEF2 activity in cardiomyocytes (Zhang et al, 2002), the Inventors tested whether CsA could block the ability of Epac to enhance MEF2 transcriptional activity. As shown in **Figure 7A**, CsA (0.5 μM) failed to inhibit Epac-induced MEF2 dependent luciferase activity indicating that calcineurin was not involved in this signaling pathway. In contrast, an inhibitor of CaMKII, KN-93 (1 μM) (Tombes et al, 1995) completely blocked the effect of EpacWT or Epac-DcAMP on MEF2-Luc activity (**Figure 7B**). These results suggest a requirement for CaMKII in Epac-induced MEF2 transcriptional activity. Accordingly, the Inventors found that Ad.Epac-DcAMP-induced increase in cell surface area was significantly decreased by KN-93 (1 μM) (**Figure 7C**).

Involvement of Rac in Epac-induced NFAT dependent cardiomyocyte hypertrophy

As Rac was found to be a downstream component of Epac signaling pathway (**Figures 1A, 1B et 1C**), the Inventors next examined the involvement of Rac in Epac-induced NFAT and MEF-2 transcriptional activities. Primary cardiomyocytes were transfected with either NFAT-Luc or MEF2-Luc, and the effect of RacS17N, a negative dominant form of Rac was tested on Epac-mediated activation of the Luc reporter constructs. Ad.RacS17N completely inhibited Epac-induced NFAT transcriptional activity (**Figure 8A**) whereas Ad.RacS17N had no effect on MEF2 transcriptional activity in cells infected with either Ad.EpacWT (**Figure 8B**) or Ad.Epac-DcAMP (data not shown). The involvement of Rac in Epac signaling pathway controlling cardiomyocyte hypertrophy was further supported by the observation that Ad.RacS17N inhibited Epac-induced ANF expression (**Figure 8C**). Consistent with these findings, Ad.RacS17N inhibited Epac-induced cytoskeletal reorganization (**Figure 9A**) and increase in cell surface areas (**Figure 9B**). Altogether, these data clearly indicate that Epac activates calcineurin/NFAT signaling pathway via the small G protein, Rac (**Figure 10**).

Epac1 is increased in patients with heart failure

To test whether the expression level of Epac is deregulated in heart failure, the Inventors performed the quantification of the major isoform of Epac in the heart, Epac1 by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in **Figure 12**, Epac1

mRNA expression is significantly increased in patients with heart failure as compared to control samples.

Discussion

The present study shows for the first time that cAMP-dependent activation of Epac induces cardiomyocyte hypertrophy in adult and neonatal rat cardiac myocytes. This is based on the observation that Epac activation leads to morphological changes, increases protein synthesis and induces alteration of gene expression of cardiac hypertrophic markers such as ANF and skeletal α-actin. In addition, the Inventors found that Epac activates a prohypertrophic signaling pathway which involves the Ca2+ sensitive phosphatase, calcineurin and its primary downstream effector, NFAT as well as MEF2. Epac-induced NFAT activation was dependent of Rac activity.

In their study, the Inventors showed that the Epac-specific cAMP analogue 8-CPT produces bursts of Ca2+ transients in neonatal myocytes. Low concentrations of 8-CPT (100 nM) elicited this effect in the presence of physiological external [Ca2+], while much higher ones (100 µM) were required in the absence of extracellular Ca2+. In other words, the apparent potency of 8-CPT to trigger intracellular Ca2+ spikes was enhanced in the presence of external Ca2+. Several explanations could be given to such an observation. A trivial one is that external Ca2+ directly facilitates 8-CPT penetration in the cell. Alternatively, these results suggest that 8-CPT acts through two distinct mechanisms, the activation of a Ca2+ influx with a high sensitivity and, with a lower sensitivity, the mobilisation of intracellular Ca2+.

In agreement with structural data (Enserink et al, 2002), the present results exclude the participation of HCN channels and PKA, the two other main cAMP targets in cardiac cells, as a mechanism of 8-CPT action on Ca2+ homeostasis. Indeed **Figures 4B and 4D** show that neither Cs+ nor the pharmacological PKA inhibitor H89 prevented 8-CPT-induced Ca2+ transients. Therefore, Epac-induced intracellular Ca2+ modulation might complement the previously reported ability of cAMP to enhance the activity of L-type Ca2+ channels and to sensitize RyR2 in a PKA-dependent manner in cardiac myocytes. The findings of the Inventors are in line with recent studies in pancreatic ß-cells and INS-1 insulin-secreting cells, demonstrating a PKA-independent mobilization of intracellular Ca2+ by the cAMP-elevating hormone glucagon-like peptide 1 and the implication of Epac in this effect (Tsuboi et al, 2003). In these cells, activation of endogenous Epac triggers Ca2+-induced Ca2+ release (Kang et al, 2003) and it is suggested that a functional coupling exists between Epac and the

RyR in these cellular systems (Holz, 2004). Therefore, one could imagine in cardiac myoyctes, that Epac might interact with Ca2+ release channels or act via an intermediary to promote kinase-mediated phosphorylation of Ca2+ handling proteins. Interestingly, the small GTPase Rap1 which is an effector of Epac is suspected to play a role in cAMP-induced [Ca2+]i increase via SERCA3b in megakaryocytes (den Dekker et al, 2002; Magnier et al, 1994). Alternatively, inositol 1,4,5-trisphosphate receptors (IP3Rs) might be involved in Epac-induced intracellular Ca2+ release since this cAMP-GEF has been previously shown to stimulate phospholipase C via the small GTPase Rap2B in HEK-293 cells and neuroblastoma cell lines (Keiper et al, 2004).

The Inventors found that the Epac-specific activator, 8-CPT induced Rac activation in primary cardiomyocytes and HL-1 cells. In addition, the Inventors showed that EpacWT or Epac-ΔcAMP expression also enhanced Rac activation in rat cardiomyocytes. This is in accordance with the recent findings of the Inventors showing that Epac induces Rac activation in a cAMP-dependent but PKA-independent manner in non-cardiac cells such as primary cortical neurons and CHO cells (Maillet et al, 2003). As the Inventors found that Rac was activated by Ca2+ following Epac stimulation, it is reasonable to think that Rac might be regulated by a GEF which is sensitive to Ca2+. Such a GEF has been reported for small GTPases of the Ras family (Keiper et al, 2004; Quilliam et al, 2002). Another molecular target which could be involved in Ca2+-dependent Rac activation is the Rho GDP-dissociation inhibitor (RhoGDI). Indeed, RhoGDI retains Rac into the cytoplasm and must dissociate to allow Rac to encounter its GEFs (Robbe et al, 2003; Schmidt & Hall, 2002). Recently, Price and colleagues (2003) have shown that Ca2+ induces a disruption of the Rac-Rho GDI complex leading to the translocation and activation of Rac in PC3 cells. Thus, one could speculate that such a mechanism might occur in cardiomyocytes and contribute to Epac-induced Ca2+-dependent Rac activation.

The Inventors report for the first time that Epac is implicated in the activation of NFAT and MEF2 in cardiac myocytes. The ability of Epac to stimulate NFAT activity was significantly inhibited by treatment with CsA and VIVIT, suggesting that calcineurin activity is regulated by Epac. Accordingly, the Inventors found that Epac up-regulates the expression of MCIP1, a well known modulator of calcineurin signaling which possesses a series of NFAT binding sites in its gene promoter (Vega et al, 2002; Yang et al, 2000). In addition, Ad.VIVIT partially reversed Epac-induced cardiomyocyte hypertrophy indicating that Epac is a new regulator of the hypertrophic calcineurin/NFAT signaling pathway. Interestingly, expression of a negative dominant form of Rac, RacS17N inhibited Epac-induced NFAT

activation but failed to do so on MEF2 transcriptional activity. In accordance with the present results, RacS17N has been shown to block NFAT activation in immune cells (Jacinto et al, 1998).

A variety of Ca2+-dependent signal transduction pathways have been implicated in cardiac hypertrophy, but whether these pathways are dependent or interdependent is unclear (Molkentin, 2004; Zhang & Brown, 2004). Passier and colleagues (2000) have reported that calcineurin/NFAT and CaMKII/MEF2 pathways act in parallel and preferentially target different transcription factors to induce cardiac hypertrophy. However, in their study, the Inventors found that both calcineurin/NFAT and CaMKII/MEF2 pathways were required to trigger the hypertrophic program in neonatal cardiomyocytes as independent inhibition of one of these two signaling cascade was sufficient to block Epac-induced hypertrophic growth (**Figures 7D, 8C, 9A**). This raises the possibility that CaMKII and calcineurin pathways converge on common downstream target genes in the hypertrophic signaling pathway initiated by Epac. In line with this hypothesis, NFAT has been shown to bind DNA in conjunction with MEF2, in promoter/enhancer regions controlling transcription of genes encoding proteins of the slow-fiber program (Chin et al, 1998). In addition, two hypertrophic signaling modules, calcineurin/NFAT and MEK1-ERK1/2 coordinately regulate cardiac growth through two distinct mechanisms (Sanna et al., 2005).

Thus, the Inventors propose a new cAMP signaling pathway in which a prolonged activation of Epac leads to a sustained increase in [Ca2+]i which then activates CaMKII and Rac. The latter increases calcineurin/NFAT activation. This signaling cascade activates hypertrophic gene expression and induces the morphological aspects of cardiac myocyte hypertrophy (**Figure 10**). These results thus open new insights into the signaling pathways by which cAMP may mediate its biological effects in cardiomyocytes and thus, raise the question of the identity of the neurohormonal factors which are involved in the regulation of Epac activity in cardiac myocytes. Finally, the fact that the Inventors found an upregulation of Epac1 gene expression in patients with heart failure suggests that this guanine nucleotide exchange factor contribute to the development of this pathology and strengthens the hypothesis that inhibitors of Epac are useful for the treatment of such cardiac pathology.

EXAMPLE 2: Construction of siRNAs that specifically inhibit the expression of Epac

To provide siRNAs that specifically inhibit the expression of Epac, the following guidelines are used according to Elbashir et al., 2002 : 1) Selection of the target region from

the open reading frame (ORF) of the cDNA sequence preferably 50 to 100 nucleotides downstream of the start codon. 2) Determination of a 21 nucleotide sequence in the target mRNA that begins with an AA dinucleotide (Elbashir et al., 2001). Thus sequences are 5'-AA(N19)UU, where N is any nucleotide. Sequences must contain approximately 50% G/C. 3) Blast-search (www.ncbi.nih.go/BLAST) the selected siRNA sequences against EST libraries or mRNA sequences of the respective organism to ensure that only a single gene is targeted. Any target sequences with more than 16 to 17 contiguous base pairs of homology to other coding sequences must be eliminated. 4) Synthesis of several siRNA sequences are advisable to control for the specificity of the knock-down experiments.

Then A) the software of Qiagen company or the software "online target finder" of the custom chemical synthesis service for siRNA present on the website of the RNA company Ambion is used to design potential sequences based on the guidelines described above.

B) Selected siRNA sequences are purchased fully deprotected, desalted with PAGE purification and delivered in dry form along with RNAse-free water and 5X annealing buffer.

C) The next step is the annealing of the siRNAs to produce siRNA duplexes ready to use for RNAi transfection experiments. Annealing can be performed as follows: incubation of equimolecular concentration of oligonucleotides (20 μM) in annealing buffer for 1 min at 90°C, centrifugation (15s) and incubation at 37°C for 1h. The siRNA duplex (20μM) is ready to use for RNAi experiments and can be stored at –20°C and undergo multiple freeze-thaw cycles.

D) Transfection of siRNA duplex is performed using the Lipofectamine 2000 (Clontech) in primary neonatal rat cardiomyocytes with optimum medium according to the manufacturer's instructions and assay for silencing is carried out 1 and 2 days after transfection. The rat neonatal cardiomyocytes are seeded the previous day in 12 well plates at 40 to 50% of confluence. Transfection efficiency is typically around 90-95%, although it is cell type dependent.

E) The effect of silencing of the several isoforms of Epac (Epac1 accession number NM_006105 of sequence SEQ ID N0 : 1, Epac2 NM_007023 of sequence SEQ ID N0 : 3 and Repac BC_039203 of sequence SEQ ID N0 : 5) is investigated on various parameters of cardiac hypertrophy (cell size, hypertrophic gene markers) upon cardiomyocytes treatment with various hypertrophic stimuli (i.e : isoproterenol, angiotensin II, phenylephrine, endotheline-1), in the aim of future applications in human cardio-physiopathology.

EXAMPLE 3: Non human model of cardiac hypertrophy induced by Epac.

Cardiac hypertrophy has been induced in mouse by the specific expression of a positive dominant form of Epac1 (Epac-ΔcAMP) in mouse cardiomyocytes. The cDNA coding for the human form of Epac1 lacking its first 322 amino acids (EpacΔcAMP) and containing a HA epitope in its N terminus (de Rooij *et al.*, *Nature* 396: 474-477) was fused to the α-Myosin Heavy Chain cardiac specific promoter (Gulick et al., 1991) (**Figure 14**). The plasmid construct containing the α-Myosin Heavy Chain promoter cloned upstream HA-Epac-ΔcAMP (**Figure 14**) is then linearized and inserted into the Hypoxanthine PhosphoRibosylTransferase (*hprt*) locus of BPES cells *hprt* negative, according to the technique described by Farhadi et al. 2003 and to the international application WO2005/005619. Positive clones are then microinjected into mouse blastocytes (C57Bl/6 genetic background), which are grafted into foster females. Offspring is screened for the presence of the transgene by Southern blotting analysis performed on DNA extracted from tail biopsies. Second generation of transgenic heterozygous animals is sacrificed to check for the RNA and the protein expressions of the transgene and its functional activity in the heart.

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CLAIMS

1. The use of at least one Epac (Exchange Protein directly Activated by cAMP) antagonist for the manufacture of a medicament intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis.

2. The use according to claim 1, wherein the antagonist is selected from the list comprising Epac activation inhibitors, Epac activity inhibitors, Epac intracellular localization disruption agents, and Epac expression inhibitors.

3. The use according to claim 1 or 2, wherein the antagonist is an Epac activation inhibitor selected from the list comprising :

- antibodies, fragments thereof, or aptamers, directed against Epac cAMP binding sites,
- cAMP analogues, such as cAMP derivatives or 8-(4-chlorophenylthio)-2'-O-methyladenosine-3'-5-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) derivatives,
- Brefeldin A or derivatives thereof.

4. The use according to claim 1 or 2, wherein the antagonist is an Epac activity inhibitor, in particular an inhibitor of the Guanine nucleotide Exchange Factor (GEF) domain of Epac, or an inhibitor of the Ras Exchange Motif (REM) domain of Epac, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against the GEF domain or the REM domain of Epac,
- Brefeldin A or derivatives thereof.

5. The use according to claim 1 or 2, wherein the antagonist is an Epac intracellular localization disruption agent, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against an Epac cellular localization domain, such as the Dishevelled Egl-10 Pleckstrin (DEP) domain,
- Brefeldin A or derivatives thereof.

6. The use according to claim 1 or 2, wherein the antagonist is an Epac expression inhibitor selected from the list comprising:

- an antisense nucleic acid directed against Epac mRNAs,
- a single stranded DNA, directed against Epac double strand DNA,
- a double stranded RNA, a siRNA or a shRNA, comprising Epac nucleic acid sequences,
- a ribozyme directed against Epac mRNAs.

7. A method for treating a pathology selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis, in a patient, comprising administering to said patient a therapeutically effective amount of at least one Epac antagonist.

8. The method according to claim 7, wherein the antagonist is selected from the list comprising Epac activation inhibitors, Epac activity inhibitors, Epac intracellular localization disruption agents, and Epac expression inhibitors.

9. The method according to claim 7, wherein the antagonist is an Epac activation inhibitor selected from the list comprising:
- antibodies, fragments thereof, or aptamers, directed against Epac cAMP binding sites,
- cAMP analogues, such as cAMP derivatives or 8-(4-chlorophenylthio)-2'-O-methyladenosine-3'-5-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) derivatives,
- Brefeldin A or derivatives thereof.

10. The method according to claim 7, wherein the antagonist is an Epac activity inhibitor, in particular an inhibitor of the Guanine nucleotide Exchange Factor (GEF) domain of Epac, or an inhibitor of the Ras Exchange Motif (REM) domain of Epac, selected from the list comprising:
- antibodies, fragments thereof, or aptamers, directed against the GEF domain or the REM domain of Epac,
- Brefeldin A or derivatives thereof.

11. The method according to claim 7, wherein the antagonist is an Epac intracellular localization disruption agent, selected from the list comprising:
- antibodies, fragments thereof, or aptamers, directed against an Epac cellular localization domain, such as the Dishevelled Egl-10 Pleckstrin (DEP) domain,
- Brefeldin A or derivatives thereof.

12. The method according to claim 7, wherein the antagonist is an Epac expression inhibitor selected from the list comprising:

- an antisense nucleic acid directed against Epac mRNAs,
- a double stranded RNA, a siRNA or a shRNA comprising Epac nucleic acid sequences,
- a ribozyme directed against Epac mRNAs.

13. Pharmaceutical compositions comprising as active substance an Epac expression inhibitor selected from the list comprising:

- an antisense nucleic acid directed against Epac mRNAs,
- a double stranded RNA, a siRNA or a shRNA comprising Epac nucleic acid sequences,
- a ribozyme directed against Epac mRNAs.

in association with a pharmaceutically acceptable carrier.

14. Pharmaceutical compositions comprising as active substance an Epac activation inhibitor, an Epac activity inhibitor, or an Epac intracellular localization disruption agent, selected from the list comprising:

- antibodies, fragments thereof, or aptamers, directed against Epac cAMP binding sites,
- antibodies, fragments thereof, or aptamers, directed against the GEF domain or the REM domain of Epac,
- antibodies, fragments thereof, or aptamers, directed against an Epac cellular localization domain, such as the DEP domain,

in association with a pharmaceutically acceptable carrier.

15. The use of Epac for screening compounds intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis.

16. A method for screening compounds intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis, comprising the steps of:

- contacting Epac with a compound to screen in the presence of cAMP or an Epac activating cAMP analogue,
- assaying Epac activation,
- selecting compounds which inhibit Epac activation, in particular compounds which inhibit at least 30% of Epac activation, as compared to Epac activation in the absence of said compounds.

17. The method according to claim 16, wherein Epac is also contacted with an effector protein liable to be activated upon Epac activation, such as Rap1, Rap2, Rac or Ras, and Epac activation is assessed by measuring the activity of said effector protein.

18. A method for screening compounds intended for the prevention or the treatment of pathologies selected from the list comprising cardiac hypertrophy, cardiac arrhythmias, valvulopathies, diastolic dysfunction, chronic heart failure, ischemic heart failure, and myocarditis comprising the steps of:
- contacting cardiomyocytes having increased Epac activity as compared to normal cardiomyocytes with compounds to screen,
- assessing the hypertrophic properties of said cardiomyocytes having increased Epac activity,
- selecting compounds which inhibit the hypertrophic properties of said cardiomyocytes having increased Epac activity, as compared to the hypertrophic properties of cardiomyocytes having increased Epac activity not treated by said compounds.

19. The method according to any of claims 16 to 18, wherein the compounds to screen are:
- cAMP analogues, such as cAMP derivatives or 8-(4-chlorophenylthio)-2'-O-methyladenosine-3'-5-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) derivatives, or
- Brefeldin A derivatives.

20. A non-human transgenic mammal for use as a model of cardiac hypertrophy, wherein Epac activity is increased with respect the corresponding wild type non-human mammal.

21. A non-human transgenic mammal for use as a model of cardiac hypertrophy according to claim 20, wherein Epac is over-expressed with respect the corresponding wild type non-human mammal, in particular said non-human transgenic mammal comprises Epac coding

sequences under the control of cardiac-specific promoters having a stronger transcription activity than Epac natural promoter, such as the promoter of the α-Myosin Heavy Chain.

22. A non-human transgenic mammal for use as a model of cardiac hypertrophy according to claim 20 or 21, wherein said non-human transgenic mammal comprises nucleic sequences encoding a constitutively activated form of Epac lacking the activating cAMP binding domain.

23. A non-human transgenic mammal for use as a model of cardiac hypertrophy according to claim 20 to 22, wherein said mammal is a mouse.

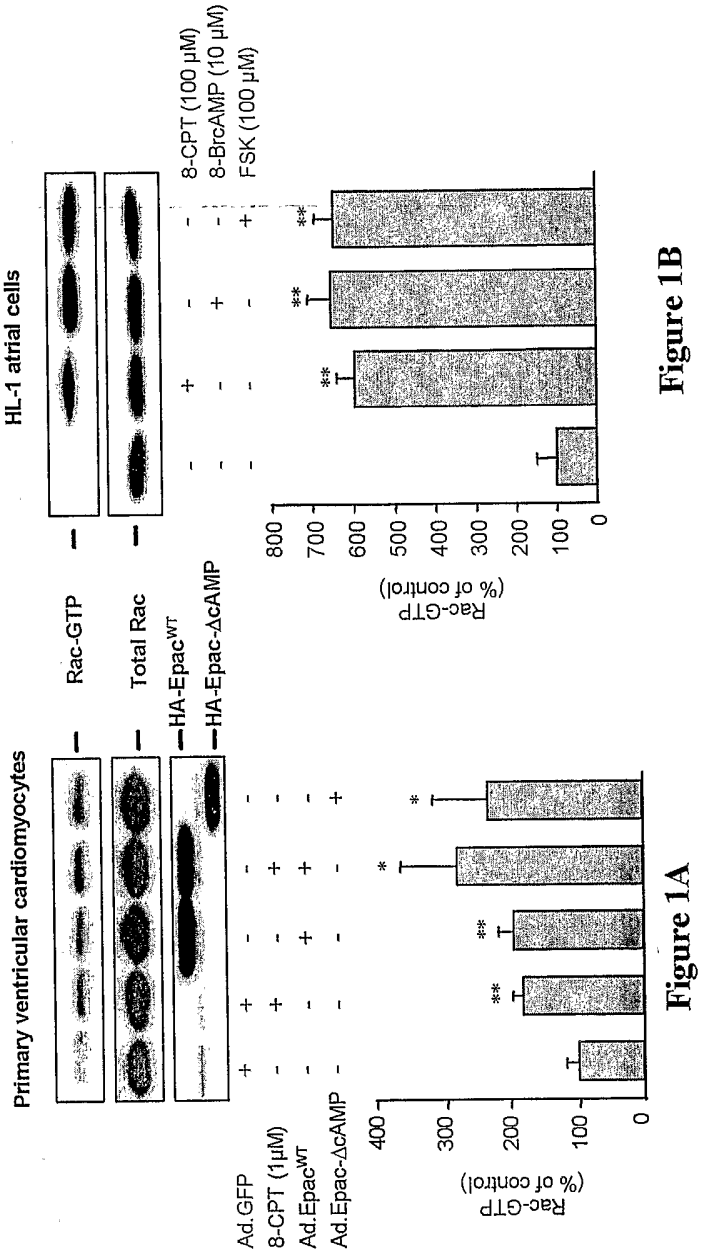


Figure 1A

Figure 1B

Figure 1C

2/16

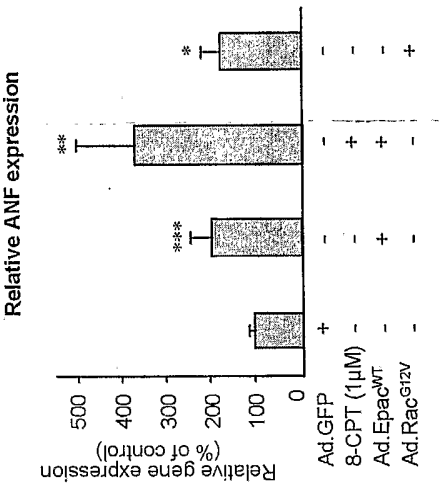


Figure 2A

Figure 2B

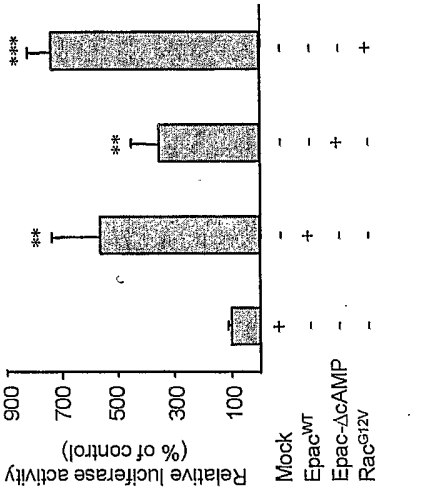


Figure 2C

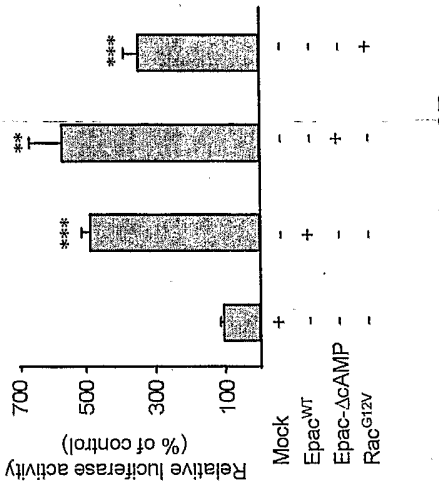


Figure 2D

3/16

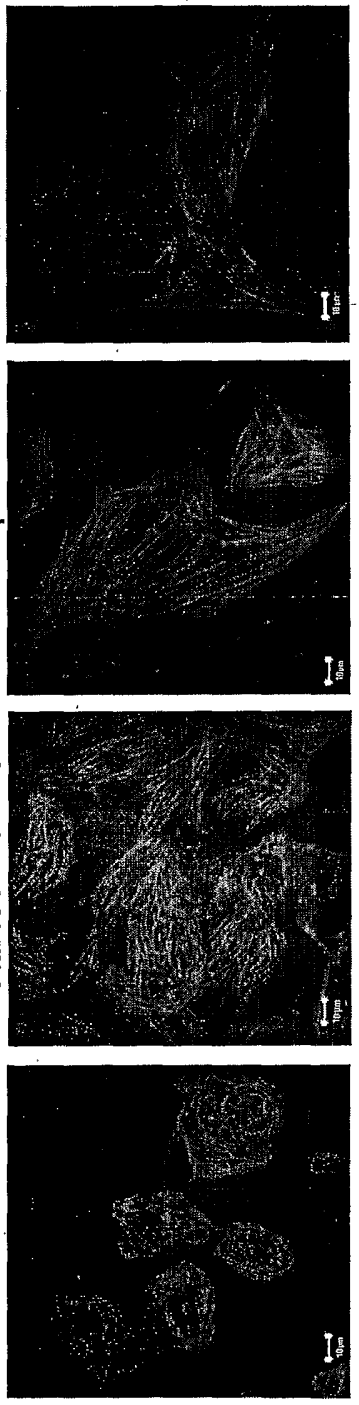


Figure 3A

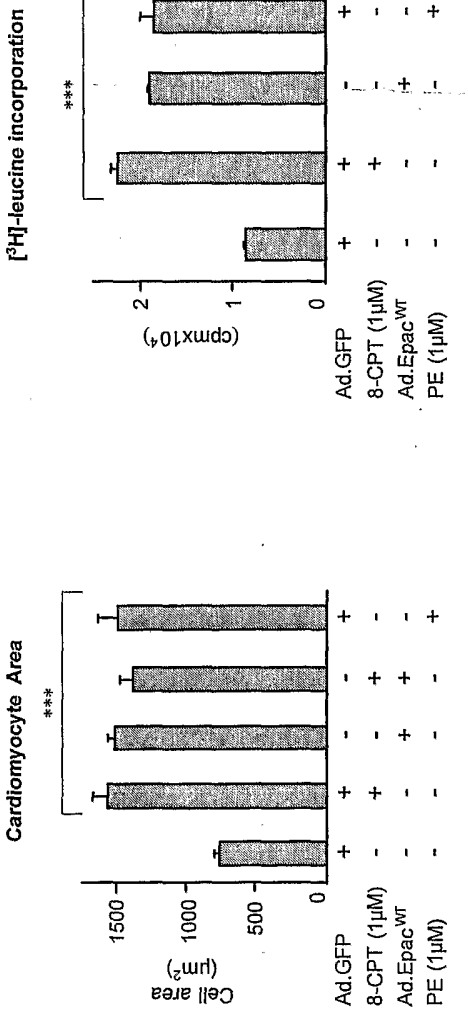
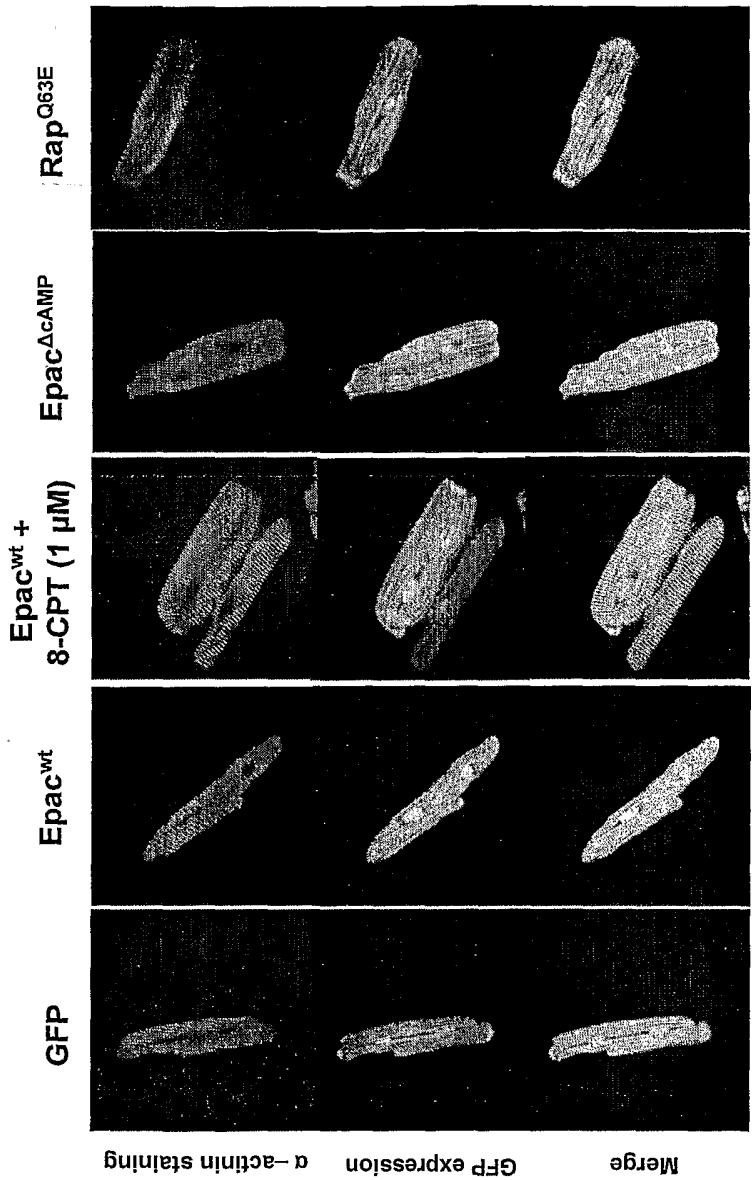


Figure 3B

Figure 3C



4/16

Figure 3D

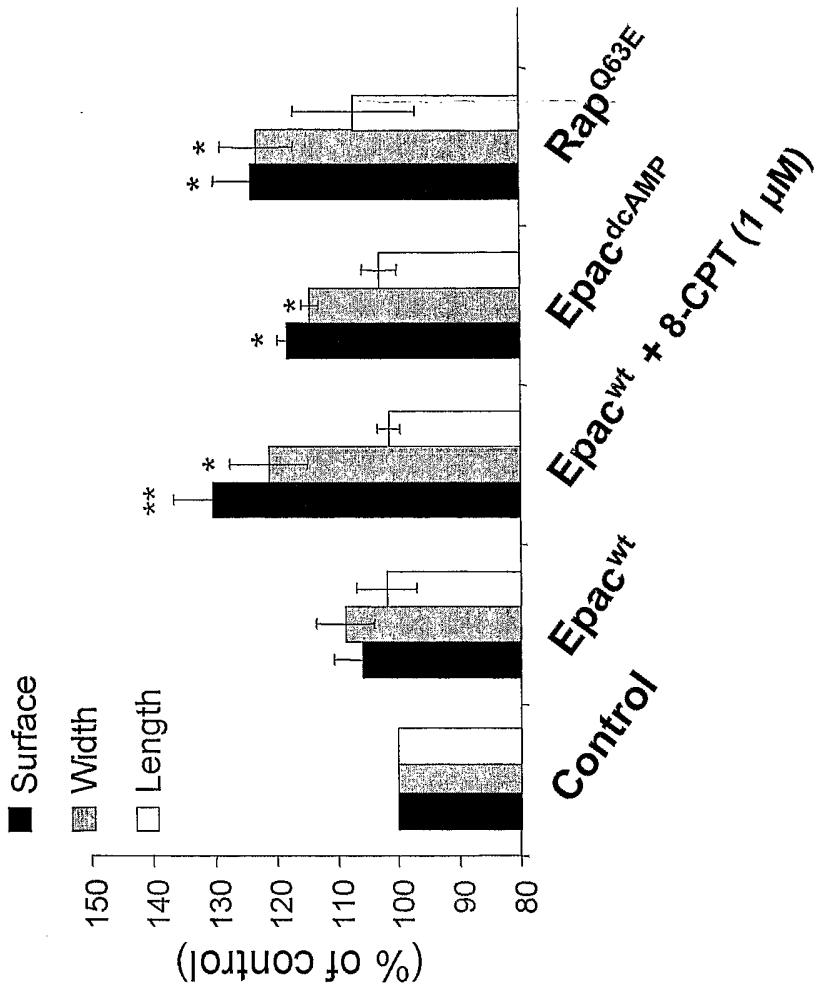

Surface
Width
Length
(% of control)
150
140
130
120
110
100
90
80
Control
Epac^wt
Epac^wt + 8-CPT (1 µM)
Epac^dcAMP
Rap^Q63E
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Figure 3E

6/16

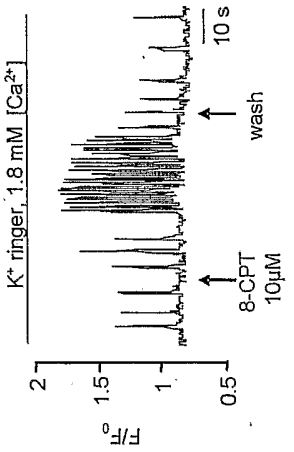


Figure 4A

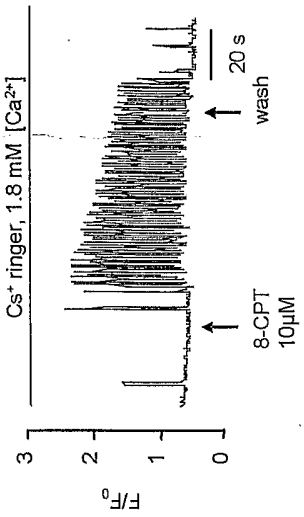


Figure 4B

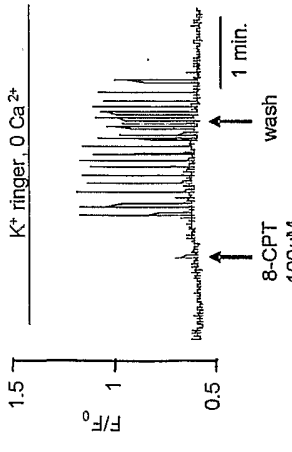


Figure 4C

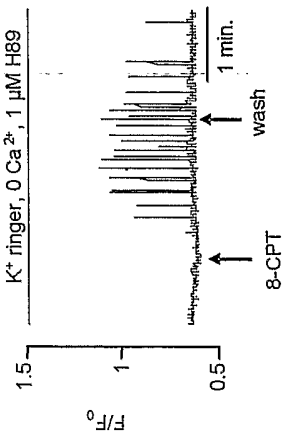


Figure 4D

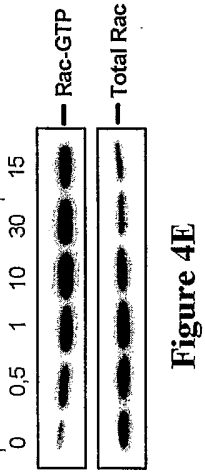


Figure 4E

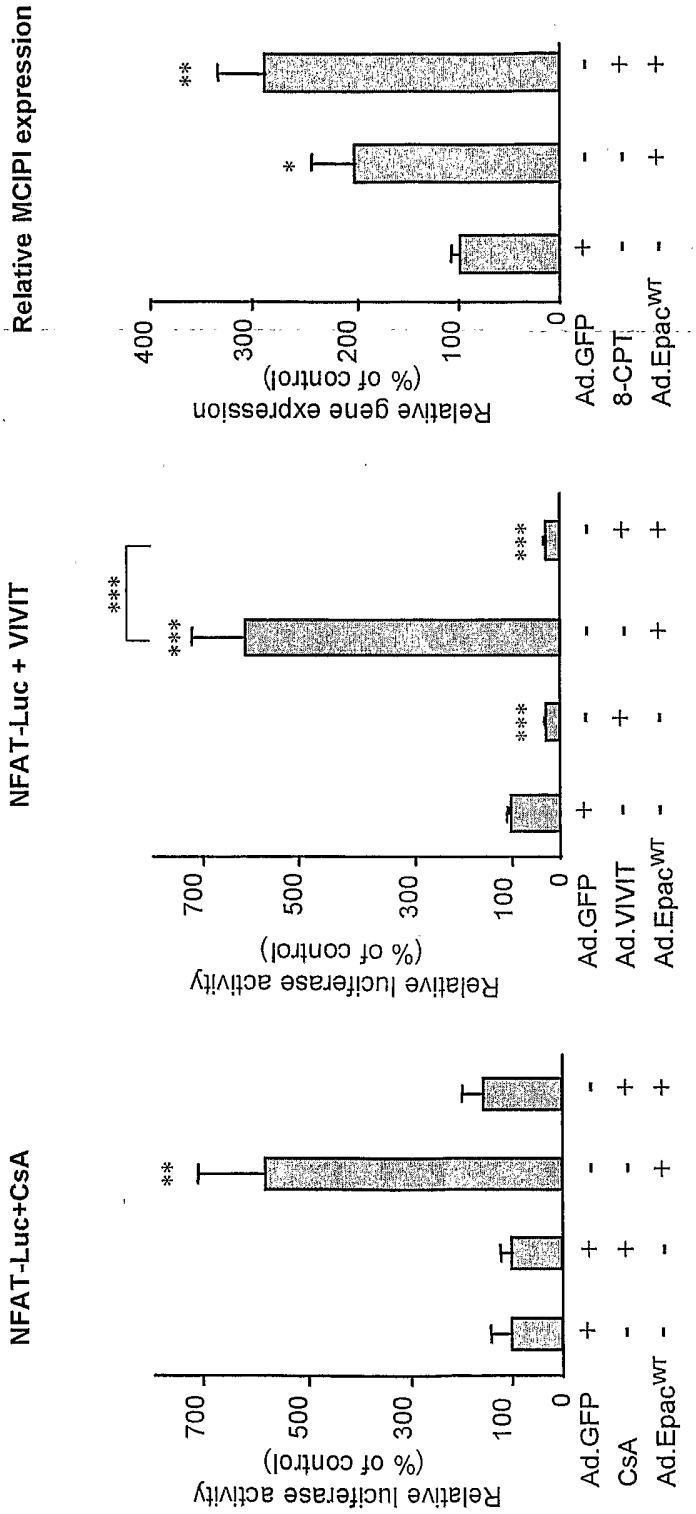


Figure 5A

Figure 5B

Figure 5C

8/16

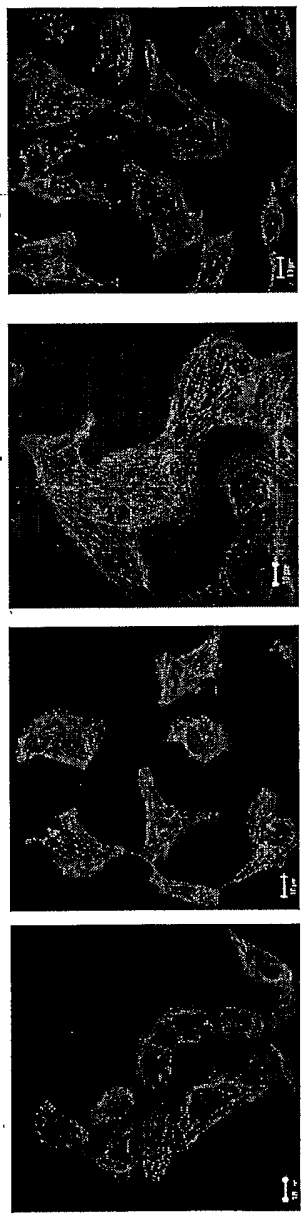


Figure 6A

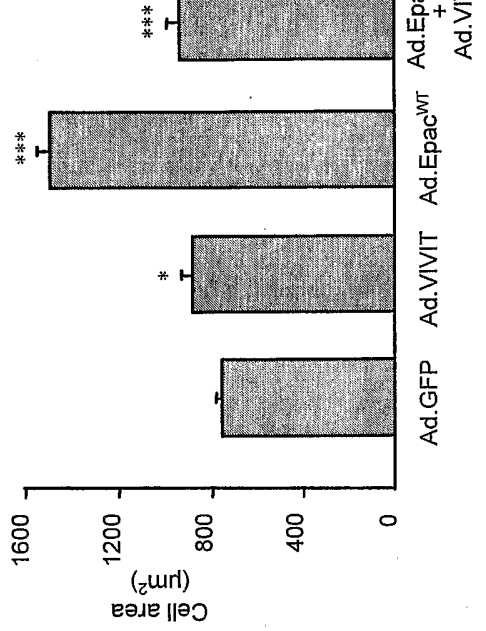


Figure 6B

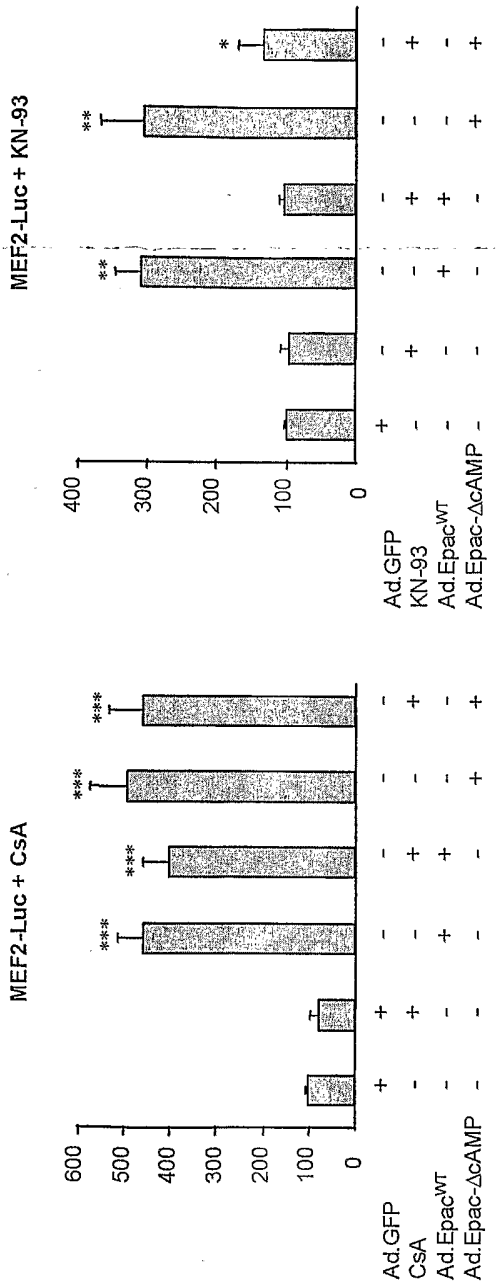


Figure 7A

Figure 7B

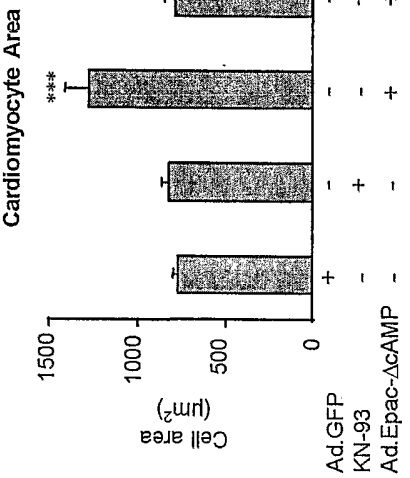
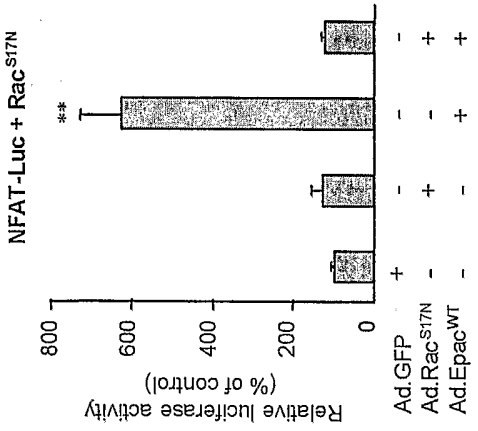


Figure 7C



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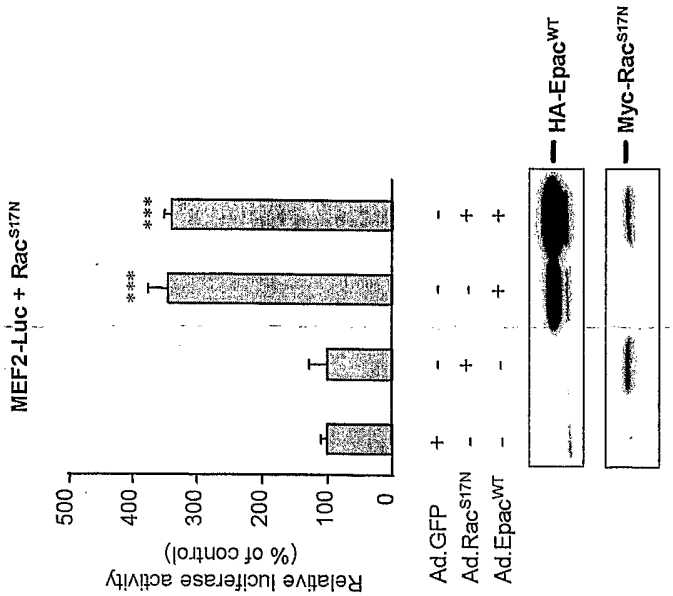

Figure 8B

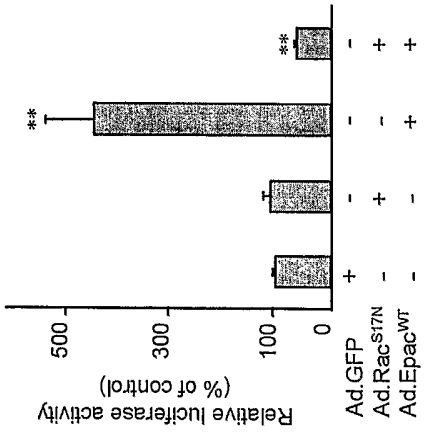

Figure 8C

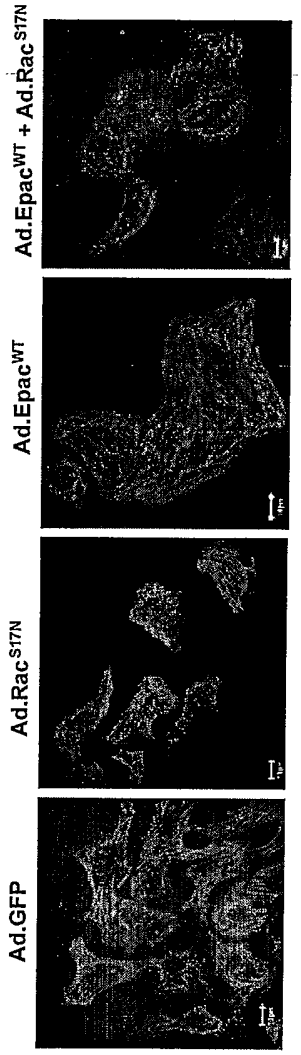

Figure 9A

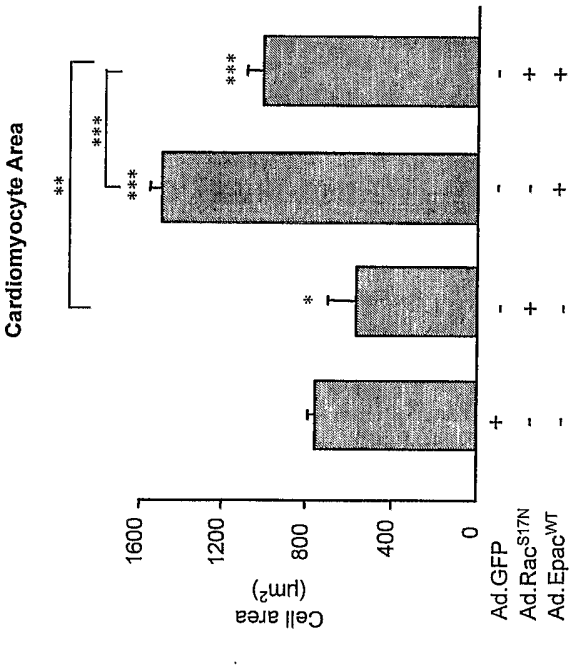

Figure 9B

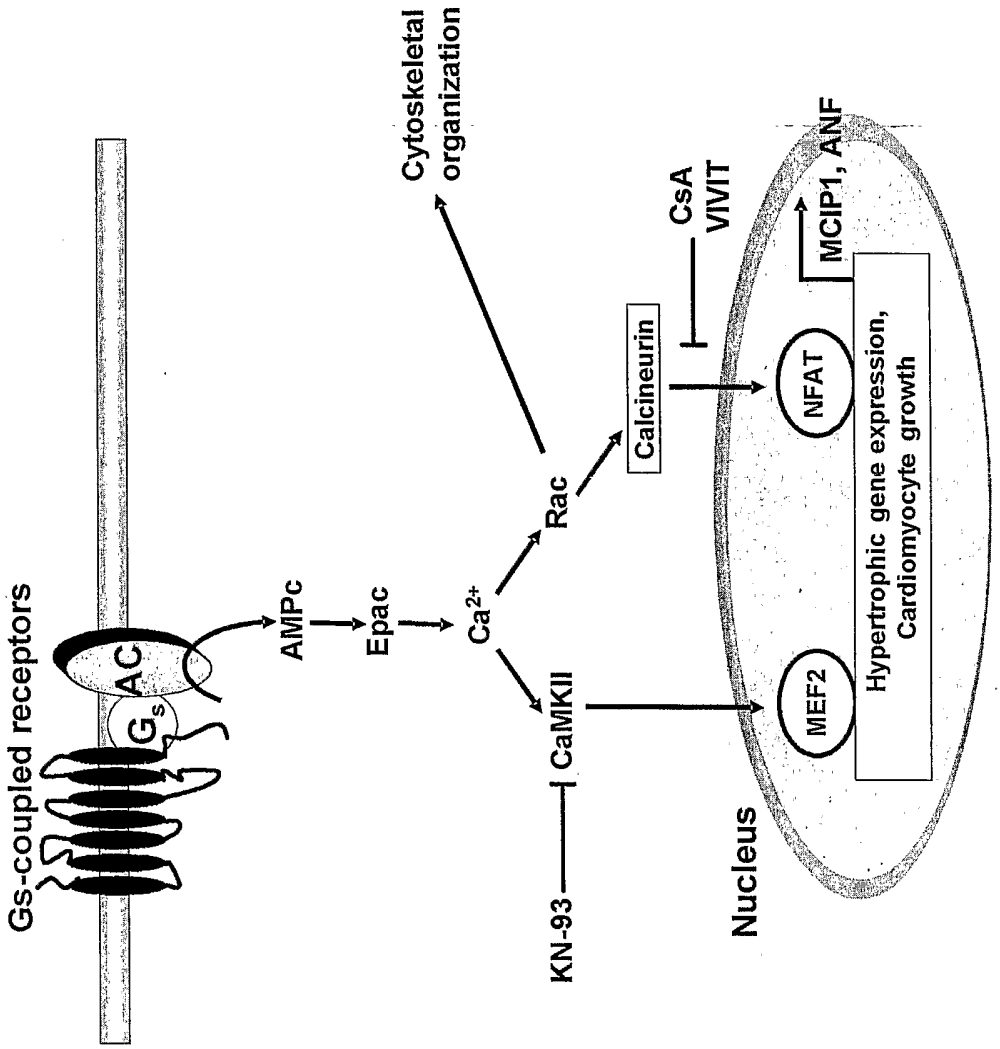
Gs-coupled receptors
AC
G_s
AMPc
Epac
Ca^{2+}
Rac
CaMKII
KN-93
Cytoskeletal organization
Calcineurin
CsA
VIVIT
NFAT
MCIP1, ANF
MEF2
Hypertrophic gene expression, Cardiomyocyte growth
Nucleus

Figure 10

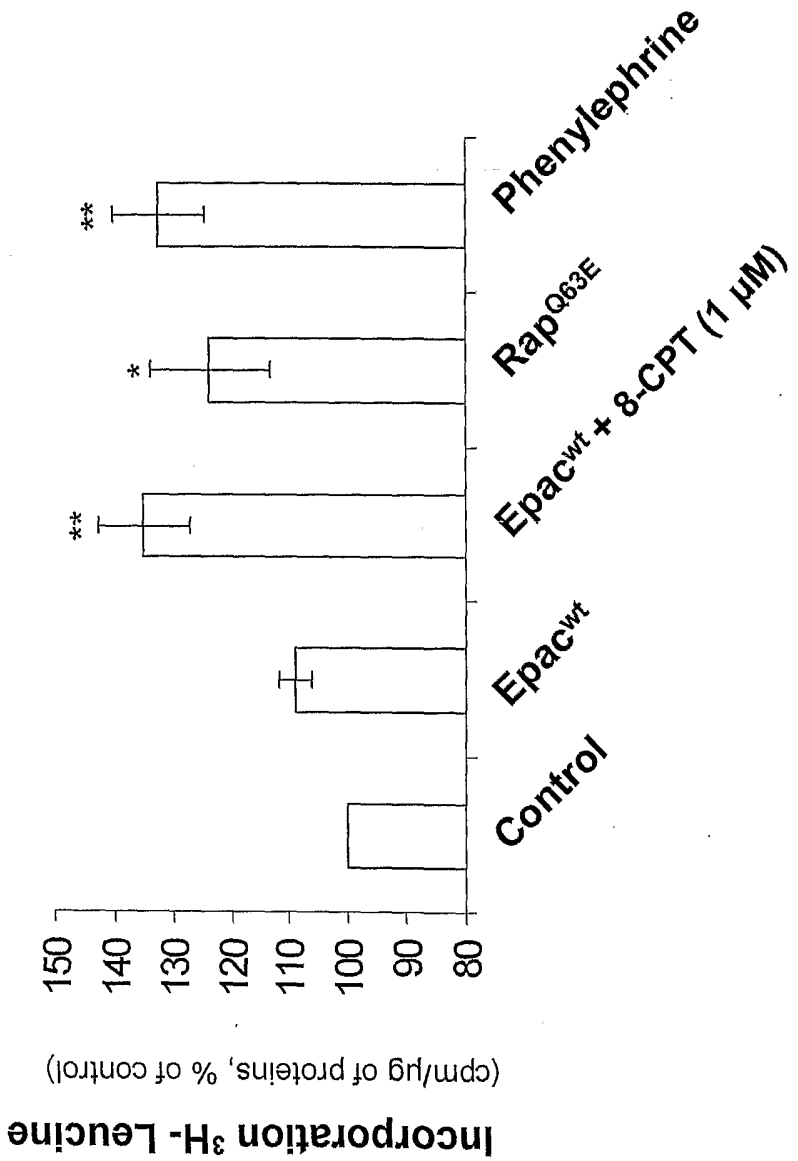
Incorporation 3H- Leucine
(cpm/µg of proteins, % of control)
150
140
130
120
110
100
90
80
Control
Epac^wt
Epac^wt + 8-CPT (1 µM)
Rap^Q63E
Phenylephrine
**
*
**

Figure 11

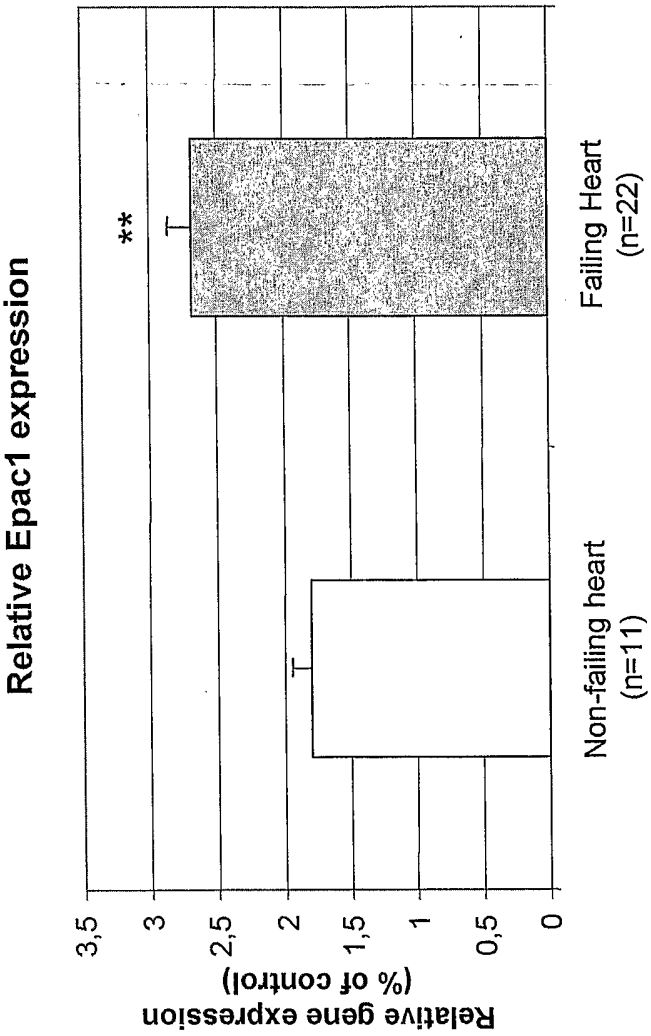
Relative Epac1 expression
**
Relative gene expression
(% of control)
3,5
3
2,5
2
1,5
1
0,5
0
Non-failing heart
(n=11)
Failing Heart
(n=22)

Figure 12

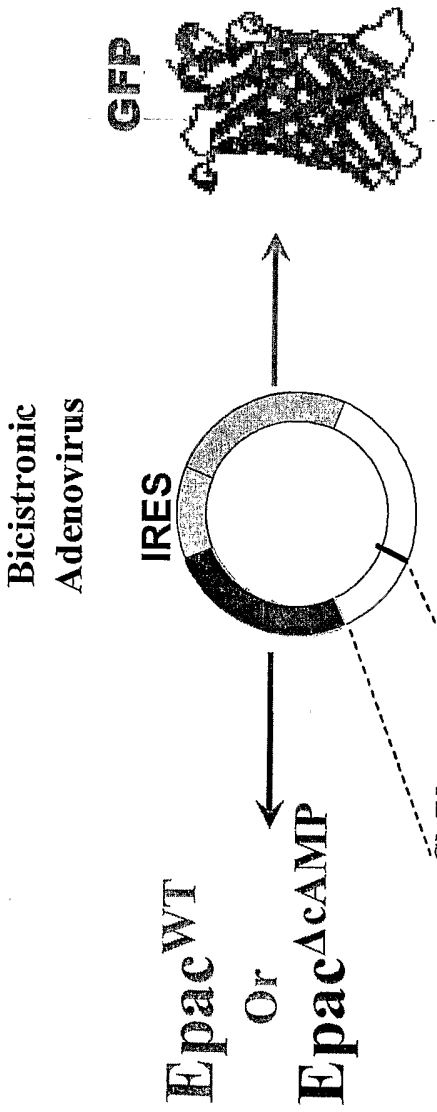
GFP
IRES
Bicistronic
Adenovirus
EpacWT
Or
EpacΔcAMP
CMV promoter

Figure 13

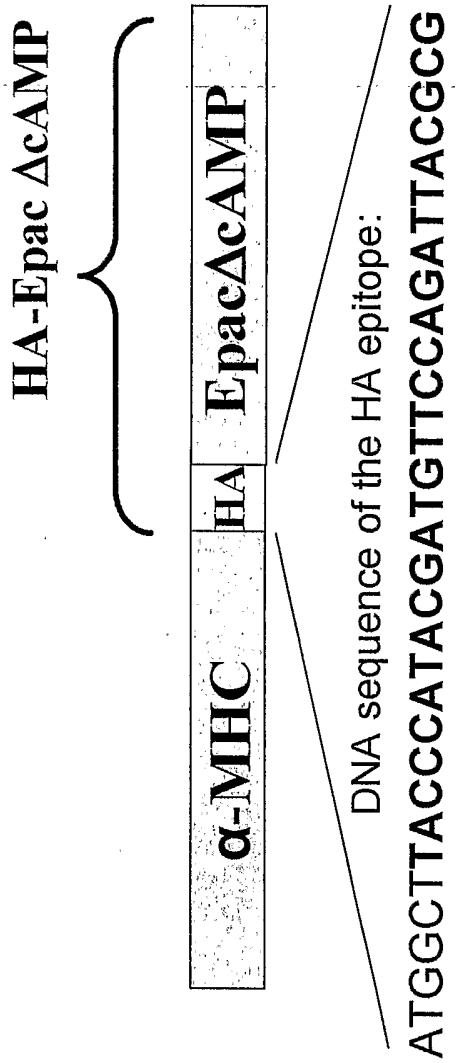
HA-Epac ΔcAMP
α-MHC
HA
EpacΔcAMP
DNA sequence of the HA epitope:
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Figure 14

SEQUENCE LISTING

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<130> WOB 05 AO INS EPAC

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<170> PatentIn version 3.1

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accctccgga tccccttatc aaagctgatg ggggtcgctg gggacccccc agccttttgc 180

tagagcctgc acggctgtgc gagcttgaaa agaaacatga aggtgggctg gccaggtgag 240

agctgctggc aggtgggcct ggctgtggag gatagcccag ctctgggagc accgcgggtg 300

ggagccctcc ctgacgtggt gccggagggg acactactca ac atg gtg ttg aga 354
Met Val Leu Arg
1

agg atg cac cgg ccc cga agc tgc tcc tac cag ctg ctg ctg gag cac 402
Arg Met His Arg Pro Arg Ser Cys Ser Tyr Gln Leu Leu Leu Glu His
5 10 15 20

cag cgt ccg agc tgc atc cag ggg ctg cgc tgg aca cca ctc acc aac 450
Gln Arg Pro Ser Cys Ile Gln Gly Leu Arg Trp Thr Pro Leu Thr Asn
25 30 35

agc gag gag tcc ctg gat ttc agc gag agc ctg gag cag gcc tcc aca 498
Ser Glu Glu Ser Leu Asp Phe Ser Glu Ser Leu Glu Gln Ala Ser Thr
40 45 50

gag cgg gtg ctc agg gct ggg agg cag ctg cat cgg cat ctg ctg gcc 546
Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Arg His Leu Leu Ala
55 60 65

acc tgc cca aac ctc atc cga gac cgg aag tac cac ctt agg ctc tat 594
Thr Cys Pro Asn Leu Ile Arg Asp Arg Lys Tyr His Leu Arg Leu Tyr
70 75 80

cgg cag tgc tgc tct ggc cgg gag ctg gtg gat ggg atc ttg gcc ctg 642
Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly Ile Leu Ala Leu
85 90 95 100

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gga ctt ggg gtc cat tcc cgg agc caa gtt gtg gga atc tgc cag gtg      690
Gly Leu Gly Val His Ser Arg Ser Gln Val Val Gly Ile Cys Gln Val
                105                 110                 115

ctg ctg gat gaa ggt gcc ctc tgc cat gtg aaa cac gac tgg gcc ttc      738
Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His Asp Trp Ala Phe
            120                 125                 130

cag gac cga gat gcc caa ttc tac cgg ttc ccc ggg ccc gag ccc gag      786
Gln Asp Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly Pro Glu Pro Glu
        135                 140                 145

ccc gtg gga act cat gag atg gag gag gag ttg gcc gaa gct gtg gcc      834
Pro Val Gly Thr His Glu Met Glu Glu Glu Leu Ala Glu Ala Val Ala
    150                 155                 160

ctg ctc tcc cag cgg ggg cct gac gcc ctg ctc act gtg gca ctt cga      882
Leu Leu Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr Val Ala Leu Arg

165                 170                 175                 180

aag ccc cca ggt cag cgc acg gat gaa gag ctg gac ctc atc ttt gag      930
Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp Leu Ile Phe Glu
                185                 190                 195

gag ctg ctg cac atc aag gct gtg gcc cac ctc tcc aac tcg gtg aag      978
Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser Asn Ser Val Lys
            200                 205                 210

cga gaa tta gcg gct gtt ctg ctc ttt gaa cca cac agc aag gca ggg     1026
Arg Glu Leu Ala Ala Val Leu Leu Phe Glu Pro His Ser Lys Ala Gly
        215                 220                 225

acc gtg ttg ttc agc cag ggg gac aag ggc act tcg tgg tac att atc     1074
Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser Trp Tyr Ile Ile
    230                 235                 240

tgg aag gga tct gtc aac gtg gtg acc cat ggc aag ggg ctg gtg acc     1122
Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys Gly Leu Val Thr
245                 250                 255                 260

acc ctg cat gag gga gat gat ttt gga cag ctg gct ctg gtg aat gat     1170
Thr Leu His Glu Gly Asp Asp Phe Gly Gln Leu Ala Leu Val Asn Asp
                265                 270                 275

gca ccc cgg gca gcc acc atc atc ctg cga gaa gac aac tgt cat ttc     1218
Ala Pro Arg Ala Ala Thr Ile Ile Leu Arg Glu Asp Asn Cys His Phe
            280                 285                 290

ctg cgt gtg gac aag cag gac ttc aac cgt atc atc aag gat gtg gag     1266
Leu Arg Val Asp Lys Gln Asp Phe Asn Arg Ile Ile Lys Asp Val Glu

        295                 300                 305

gca aag acc atg cgg ctg gaa gaa cat ggc aaa gtg gtg ctg gtg ctg     1314
Ala Lys Thr Met Arg Leu Glu Glu His Gly Lys Val Val Leu Val Leu
    310                 315                 320

gag aga gcc tct cag ggc gcc ggc cct tcc cga ccc cca acc cca ggc     1362
Glu Arg Ala Ser Gln Gly Ala Gly Pro Ser Arg Pro Pro Thr Pro Gly
```

```
325                 330                 335                 340

agg aac cgg tat aca gtg atg tct ggc acc cca gag aag atc cta gag     1410
Arg Asn Arg Tyr Thr Val Met Ser Gly Thr Pro Glu Lys Ile Leu Glu
                345                 350                 355

ctt ctg ttg gag gcc atg gga cca gat tcc agt gct cat gac cca aca     1458
Leu Leu Leu Glu Ala Met Gly Pro Asp Ser Ser Ala His Asp Pro Thr
            360                 365                 370

gag aca ttc ctc agc gac ttc ctc ctg acc cac agg gtc ttc atg ccc     1506
Glu Thr Phe Leu Ser Asp Phe Leu Leu Thr His Arg Val Phe Met Pro
        375                 380                 385

agc gcc caa ctc tgc gct gcc ctt ctg cac cac ttc cat gtg gag cct     1554
Ser Ala Gln Leu Cys Ala Ala Leu Leu His His Phe His Val Glu Pro
    390                 395                 400

gcg ggt ggc agc gag cag gag cgc agc acc tac gtc tgc aac aag agg     1602
Ala Gly Gly Ser Glu Gln Glu Arg Ser Thr Tyr Val Cys Asn Lys Arg
405                 410                 415                 420

cag cag atc ttg cgg ctg gtc agc cag tgg gtg gcc ctg tat ggc tcc     1650
Gln Gln Ile Leu Arg Leu Val Ser Gln Trp Val Ala Leu Tyr Gly Ser

                425                 430                 435

atg ctc cac act gac cct gtg gcc acc agc ttc ctc cag aaa ctc tca     1698
Met Leu His Thr Asp Pro Val Ala Thr Ser Phe Leu Gln Lys Leu Ser
            440                 445                 450

gac ctg gtg ggc agg gac acc cga ctc agc aac ctg ctg agg gag cag     1746
Asp Leu Val Gly Arg Asp Thr Arg Leu Ser Asn Leu Leu Arg Glu Gln
        455                 460                 465

tgg cca gag agg cgg cga tgc cac agg ttg gag aat ggc tgt ggg aat     1794
Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn Gly Cys Gly Asn
    470                 475                 480

gca tct cct cag atg aag gcc cgg aac ttg cct gtt tgg ctc ccc aac     1842
Ala Ser Pro Gln Met Lys Ala Arg Asn Leu Pro Val Trp Leu Pro Asn
485                 490                 495                 500

cag gac gag ccc ctt cct ggc agc agc tgt gcc atc caa gtt ggg gat     1890
Gln Asp Glu Pro Leu Pro Gly Ser Ser Cys Ala Ile Gln Val Gly Asp
                505                 510                 515

aaa gtc ccc tat gac atc tgc cgg cca gac cac tca gtg ttg acc ctg     1938
Lys Val Pro Tyr Asp Ile Cys Arg Pro Asp His Ser Val Leu Thr Leu
            520                 525                 530

cag ctg cct gtg aca gcc tcc gtg aga gag gtg atg gca gcg ttg gcc     1986
Gln Leu Pro Val Thr Ala Ser Val Arg Glu Val Met Ala Ala Leu Ala
        535                 540                 545

cag gag gat ggc tgg acc aag ggg cag gtg ctg gtg aag gtc aat tct     2034
Gln Glu Asp Gly Trp Thr Lys Gly Gln Val Leu Val Lys Val Asn Ser

    550                 555                 560

gca ggt gat gcc att ggc ctg cag cca gat gcc cgt ggt gtg gcc aca     2082
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```
Ala Gly Asp Ala Ile Gly Leu Gln Pro Asp Ala Arg Gly Val Ala Thr
565                 570                 575                 580

tct ctg ggg ctc aat gag cgt ctc ttt gtt gtc aac cca cag gaa gtg     2130
Ser Leu Gly Leu Asn Glu Arg Leu Phe Val Val Asn Pro Gln Glu Val
                585                 590                 595

cat gag ctg atc cca cac cct gac cag ctg ggg ccc act gtg ggc tct     2178
His Glu Leu Ile Pro His Pro Asp Gln Leu Gly Pro Thr Val Gly Ser
            600                 605                 610

gct gag ggg ctg gac ctg gtg agt gcc aag gac ctg gca ggc cag ctg     2226
Ala Glu Gly Leu Asp Leu Val Ser Ala Lys Asp Leu Ala Gly Gln Leu
        615                 620                 625

acg gac cac gac tgg agc ctc ttc aac agt atc cac cag gtg gag ctg     2274
Thr Asp His Asp Trp Ser Leu Phe Asn Ser Ile His Gln Val Glu Leu
    630                 635                 640

atc cac tat gtg ctg ggc ccc cag cat ctg cgg gat gtc acc acc gcc     2322
Ile His Tyr Val Leu Gly Pro Gln His Leu Arg Asp Val Thr Thr Ala
645                 650                 655                 660

aac ctg gag cgc ttc atg cgc cgc ttc aat gag ctg cag tac tgg gtg     2370
Asn Leu Glu Arg Phe Met Arg Arg Phe Asn Glu Leu Gln Tyr Trp Val
                665                 670                 675

gcc acc gag ctg tgt ctc tgc ccc gtg ccc ggc ccc cgg gcc cag ctg     2418
Ala Thr Glu Leu Cys Leu Cys Pro Val Pro Gly Pro Arg Ala Gln Leu

            680                 685                 690

ctc agg aag ttc att aag ctg gcg gcc cac ctc aag gag cag aag aat     2466
Leu Arg Lys Phe Ile Lys Leu Ala Ala His Leu Lys Glu Gln Lys Asn
        695                 700                 705

ctc aat tcc ttc ttt gcc gtc atg ttt ggc ctc agc aac tcg gcc atc     2514
Leu Asn Ser Phe Phe Ala Val Met Phe Gly Leu Ser Asn Ser Ala Ile
    710                 715                 720

agc cgc cta gcc cac acc tgg gag cgg ctg cct cac aaa gtc cgg aag     2562
Ser Arg Leu Ala His Thr Trp Glu Arg Leu Pro His Lys Val Arg Lys
725                 730                 735                 740

ctg tac tcc gcc ctc gag agg ctg ctg gat ccc tca tgg aac cac cgg     2610
Leu Tyr Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser Trp Asn His Arg
                745                 750                 755

gta tac cga ctg gcc ctc gcc aag ctc tcc cct cct gtc atc ccc ttc     2658
Val Tyr Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro Val Ile Pro Phe
            760                 765                 770

atg ccc ctt ctt ctc aaa gac atg acc ttc att cat gag gga aac cac     2706
Met Pro Leu Leu Leu Lys Asp Met Thr Phe Ile His Glu Gly Asn His
        775                 780                 785

aca cta gtg gag aat ctc atc aac ttt gag aag atg aga atg atg gcc     2754
Thr Leu Val Glu Asn Leu Ile Asn Phe Glu Lys Met Arg Met Met Ala
    790                 795                 800

aga gcc gcg cgg atg ctg cac cac tgc cga agc cac aac cct gtg cct     2802
```

```
Arg Ala Ala Arg Met Leu His His Cys Arg Ser His Asn Pro Val Pro

805                 810                 815                 820

ctc tca cca ctc aga agc cga gtt tcc cac ctc cac gag gac agc cag     2850
Leu Ser Pro Leu Arg Ser Arg Val Ser His Leu His Glu Asp Ser Gln
                825                 830                 835

gtg gcg agg att tcc aca tgc tcg gag cag tcc ctg agc acc cgg agt     2898
Val Ala Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu Ser Thr Arg Ser
            840                 845                 850

cca gcc agc acc tgg gct tat gtc cag cag ctg aag gtc att gac aac     2946
Pro Ala Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys Val Ile Asp Asn
        855                 860                 865

cag cgg gaa ctc tcc cgc ctc tcc cga gag ctg gag cca tga             2988
Gln Arg Glu Leu Ser Arg Leu Ser Arg Glu Leu Glu Pro
    870                 875                 880

ggaggggctg ggactggagc tggagcaggc acttgcagcc gggaaagcca gggtgtgccg   3048

ggccaagata ctcacaggct ggccacagct gggcaaggct ctccgtggag tggactcgag   3108

tccctggagc aggcagtgtg gaggcagcca tcccctgtga tgactggcag ctaaggagga   3168

cctcggagtg gacccgagcc aggaataacg aatgacccaa ggccaaggaa gggaggacag   3228

agaggcccca ggagtgggtg gagagtggag tgcgctggga cgttgtgtgc aatagagagg   3288

tctccacacc agatgtcttc cagaaaaaaa aaaaaaaaa                          3327
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Pro Leu Thr Asn Ser Glu Glu Ser Leu Asp Phe Ser Glu Ser Leu Glu
        35                  40                  45

Gln Ala Ser Thr Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Arg
    50                  55                  60

His Leu Leu Ala Thr Cys Pro Asn Leu Ile Arg Asp Arg Lys Tyr His
65                  70                  75                  80

Leu Arg Leu Tyr Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly
```

85 90 95

Ile Leu Ala Leu Gly Leu Gly Val His Ser Arg Ser Gln Val Val Gly
100 105 110

Ile Cys Gln Val Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His
115 120 125

Asp Trp Ala Phe Gln Asp Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly
130 135 140

Pro Glu Pro Glu Pro Val Gly Thr His Glu Met Glu Glu Glu Leu Ala
145 150 155 160

Glu Ala Val Ala Leu Leu Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr
165 170 175

Val Ala Leu Arg Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp
180 185 190

Leu Ile Phe Glu Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser
195 200 205

Asn Ser Val Lys Arg Glu Leu Ala Ala Val Leu Leu Phe Glu Pro His
210 215 220

Ser Lys Ala Gly Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser
225 230 235 240

Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys
245 250 255

Gly Leu Val Thr Thr Leu His Glu Gly Asp Asp Phe Gly Gln Leu Ala
260 265 270

Leu Val Asn Asp Ala Pro Arg Ala Ala Thr Ile Ile Leu Arg Glu Asp
275 280 285

Asn Cys His Phe Leu Arg Val Asp Lys Gln Asp Phe Asn Arg Ile Ile
290 295 300

Lys Asp Val Glu Ala Lys Thr Met Arg Leu Glu Glu His Gly Lys Val
305 310 315 320

Val Leu Val Leu Glu Arg Ala Ser Gln Gly Ala Gly Pro Ser Arg Pro
325 330 335

```
Pro Thr Pro Gly Arg Asn Arg Tyr Thr Val Met Ser Gly Thr Pro Glu
            340                 345                 350

Lys Ile Leu Glu Leu Leu Leu Glu Ala Met Gly Pro Asp Ser Ser Ala
        355                 360                 365

His Asp Pro Thr Glu Thr Phe Leu Ser Asp Phe Leu Leu Thr His Arg
    370                 375                 380

Val Phe Met Pro Ser Ala Gln Leu Cys Ala Ala Leu Leu His His Phe
385                 390                 395                 400

His Val Glu Pro Ala Gly Gly Ser Glu Gln Glu Arg Ser Thr Tyr Val
                405                 410                 415

Cys Asn Lys Arg Gln Gln Ile Leu Arg Leu Val Ser Gln Trp Val Ala
            420                 425                 430

Leu Tyr Gly Ser Met Leu His Thr Asp Pro Val Ala Thr Ser Phe Leu
        435                 440                 445

Gln Lys Leu Ser Asp Leu Val Gly Arg Asp Thr Arg Leu Ser Asn Leu
    450                 455                 460

Leu Arg Glu Gln Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn
465                 470                 475                 480

Gly Cys Gly Asn Ala Ser Pro Gln Met Lys Ala Arg Asn Leu Pro Val
                485                 490                 495

Trp Leu Pro Asn Gln Asp Glu Pro Leu Pro Gly Ser Ser Cys Ala Ile
            500                 505                 510

Gln Val Gly Asp Lys Val Pro Tyr Asp Ile Cys Arg Pro Asp His Ser
        515                 520                 525

Val Leu Thr Leu Gln Leu Pro Val Thr Ala Ser Val Arg Glu Val Met
    530                 535                 540

Ala Ala Leu Ala Gln Glu Asp Gly Trp Thr Lys Gly Gln Val Leu Val
545                 550                 555                 560

Lys Val Asn Ser Ala Gly Asp Ala Ile Gly Leu Gln Pro Asp Ala Arg
                565                 570                 575
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Gly Val Ala Thr Ser Leu Gly Leu Asn Glu Arg Leu Phe Val Val Asn
            580                 585                 590

Pro Gln Glu Val His Glu Leu Ile Pro His Pro Asp Gln Leu Gly Pro
        595                 600                 605

Thr Val Gly Ser Ala Glu Gly Leu Asp Leu Val Ser Ala Lys Asp Leu
    610                 615                 620

Ala Gly Gln Leu Thr Asp His Asp Trp Ser Leu Phe Asn Ser Ile His
625                 630                 635                 640

Gln Val Glu Leu Ile His Tyr Val Leu Gly Pro Gln His Leu Arg Asp
                645                 650                 655

Val Thr Thr Ala Asn Leu Glu Arg Phe Met Arg Arg Phe Asn Glu Leu
            660                 665                 670

Gln Tyr Trp Val Ala Thr Glu Leu Cys Leu Cys Pro Val Pro Gly Pro
        675                 680                 685

Arg Ala Gln Leu Leu Arg Lys Phe Ile Lys Leu Ala Ala His Leu Lys
    690                 695                 700

Glu Gln Lys Asn Leu Asn Ser Phe Phe Ala Val Met Phe Gly Leu Ser
705                 710                 715                 720

Asn Ser Ala Ile Ser Arg Leu Ala His Thr Trp Glu Arg Leu Pro His
                725                 730                 735

Lys Val Arg Lys Leu Tyr Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser
            740                 745                 750

Trp Asn His Arg Val Tyr Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro
        755                 760                 765

Val Ile Pro Phe Met Pro Leu Leu Leu Lys Asp Met Thr Phe Ile His
    770                 775                 780

Glu Gly Asn His Thr Leu Val Glu Asn Leu Ile Asn Phe Glu Lys Met
785                 790                 795                 800

Arg Met Met Ala Arg Ala Ala Arg Met Leu His His Cys Arg Ser His
                805                 810                 815
```

```
Asn Pro Val Pro Leu Ser Pro Leu Arg Ser Arg Val Ser His Leu His
            820                 825                 830

Glu Asp Ser Gln Val Ala Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu
        835                 840                 845

Ser Thr Arg Ser Pro Ala Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys
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Val Ile Asp Asn Gln Arg Glu Leu Ser Arg Leu Ser Arg Glu Leu Glu
865                 870                 875                 880

Pro
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                                                    Met Val Ala
                                                    1

gcg cac gct gcc cat tct tcc tcc tct gcc gag tgg atc gcc tgc ctg      165
Ala His Ala Ala His Ser Ser Ser Ser Ala Glu Trp Ile Ala Cys Leu
    5                   10                  15

gat aaa aga cca ctg gag cga tcc agc gaa gat gtg gat ata atc ttc      213
Asp Lys Arg Pro Leu Glu Arg Ser Ser Glu Asp Val Asp Ile Ile Phe
20                  25                  30                  35

act cga ctg aaa gaa gtt aaa gct ttt gag aaa ttt cac cca aat ctc      261
Thr Arg Leu Lys Glu Val Lys Ala Phe Glu Lys Phe His Pro Asn Leu
                40                  45                  50

ctt cat cag att tgc tta tgt ggt tat tat gag aat ctg gaa aag gga      309
Leu His Gln Ile Cys Leu Cys Gly Tyr Tyr Glu Asn Leu Glu Lys Gly
            55                  60                  65

ata aca tta ttt cgc cag ggt gat att gga aca aac tgg tat gct gtc      357
Ile Thr Leu Phe Arg Gln Gly Asp Ile Gly Thr Asn Trp Tyr Ala Val
        70                  75                  80

ctg gca ggg tct ttg gat gtt aaa gta tct gag acc agc agt cac cag      405
Leu Ala Gly Ser Leu Asp Val Lys Val Ser Glu Thr Ser Ser His Gln
    85                  90                  95
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gat gct gtg acc atc tgt acc ctg gga att ggg acg gcc ttt gga gag      453
Asp Ala Val Thr Ile Cys Thr Leu Gly Ile Gly Thr Ala Phe Gly Glu
100                 105                 110                 115

tcc att ctg gac aac aca ccc cgc cat gca acc atc gtt acc agg gag      501
Ser Ile Leu Asp Asn Thr Pro Arg His Ala Thr Ile Val Thr Arg Glu
                120                 125                 130

agc agt gaa ctg ctc cgc atc gag cag aag gac ttc aag gca cta tgg      549
Ser Ser Glu Leu Leu Arg Ile Glu Gln Lys Asp Phe Lys Ala Leu Trp
            135                 140                 145

gag aaa tat cga cag tat atg gca gga ctt ctg gct cct cct tat ggt      597
Glu Lys Tyr Arg Gln Tyr Met Ala Gly Leu Leu Ala Pro Pro Tyr Gly
        150                 155                 160

gtt atg gaa acg ggc tct aac aat gac agg att cct gac aag gag aac      645
Val Met Glu Thr Gly Ser Asn Asn Asp Arg Ile Pro Asp Lys Glu Asn
    165                 170                 175

aca cct ctc att gaa cct cac gtt cct ctt cgt cct gct aac acc att      693
Thr Pro Leu Ile Glu Pro His Val Pro Leu Arg Pro Ala Asn Thr Ile
180                 185                 190                 195

acc aag gtc cct tca gag aag atc ctc aga gct gga aaa att tta cga      741
Thr Lys Val Pro Ser Glu Lys Ile Leu Arg Ala Gly Lys Ile Leu Arg
                200                 205                 210

aat gcc att ctc tct cga gca cct cac atg ata aga gat aga aaa tac      789
Asn Ala Ile Leu Ser Arg Ala Pro His Met Ile Arg Asp Arg Lys Tyr
            215                 220                 225

cac cta aag aca tac aga caa tgc tgt gtg gga act gaa ctg gtg gac      837
His Leu Lys Thr Tyr Arg Gln Cys Cys Val Gly Thr Glu Leu Val Asp
        230                 235                 240

tgg atg atg cag cag aca cca tgt gtt cac tcc cgg act caa gct gtt      885
Trp Met Met Gln Gln Thr Pro Cys Val His Ser Arg Thr Gln Ala Val
    245                 250                 255

ggc atg tgg caa gtc ctg tta gaa gat ggt gtt ctc aac cac gtg gac      933
Gly Met Trp Gln Val Leu Leu Glu Asp Gly Val Leu Asn His Val Asp
260                 265                 270                 275

cag gag cac cat ttc caa gac aaa tat tta ttc tat cga ttt ctg gat      981
Gln Glu His His Phe Gln Asp Lys Tyr Leu Phe Tyr Arg Phe Leu Asp
                280                 285                 290

gat gag cac gag gat gcc cct ttg cct act gag gag gag aag aag gag     1029
Asp Glu His Glu Asp Ala Pro Leu Pro Thr Glu Glu Glu Lys Lys Glu
            295                 300                 305

tgt gat gag gag ctc cag gac acc atg ctg ctg ctg tca cag atg ggc     1077
Cys Asp Glu Glu Leu Gln Asp Thr Met Leu Leu Leu Ser Gln Met Gly
        310                 315                 320

ccc gac gcc cac atg agg atg atc ctt cgc aaa cca cct ggc cag agg     1125
Pro Asp Ala His Met Arg Met Ile Leu Arg Lys Pro Pro Gly Gln Arg
    325                 330                 335

act gtg gat gac cta gag att atc tat gag gag ctt ctt cat att aaa     1173
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Thr Val Asp Asp Leu Glu Ile Ile Tyr Glu Glu Leu Leu His Ile Lys
340                 345                 350                 355

gcc tta tcc cat ctt tct acc aca gtg aaa cga gag tta gca ggt gtt    1221
Ala Leu Ser His Leu Ser Thr Thr Val Lys Arg Glu Leu Ala Gly Val
                360                 365                 370

ctc att ttt gag tct cac gcc aaa gga ggg act gtg ttg ttt aac cag    1269
Leu Ile Phe Glu Ser His Ala Lys Gly Gly Thr Val Leu Phe Asn Gln
            375                 380                 385

ggg gaa gaa ggt acc tcc tgg tac att att cta aaa gga tca gtg aat    1317
Gly Glu Glu Gly Thr Ser Trp Tyr Ile Ile Leu Lys Gly Ser Val Asn
        390                 395                 400

gta gtc att tac ggc aag ggt gtg gtc tgc acc ctg cat gaa gga gat    1365
Val Val Ile Tyr Gly Lys Gly Val Val Cys Thr Leu His Glu Gly Asp
    405                 410                 415

gac ttc ggc aag tta gca cta gtg aat gat gcc cca cga gct gcc tct    1413
Asp Phe Gly Lys Leu Ala Leu Val Asn Asp Ala Pro Arg Ala Ala Ser
420                 425                 430                 435

atc gtc tta cga gaa gat aac tgc cat ttc tta aga gta gac aag gag    1461
Ile Val Leu Arg Glu Asp Asn Cys His Phe Leu Arg Val Asp Lys Glu
                440                 445                 450

gat ttc aac cgg atc cta agg gac gtg gag gcg aat aca gtc aga ctt    1509
Asp Phe Asn Arg Ile Leu Arg Asp Val Glu Ala Asn Thr Val Arg Leu
            455                 460                 465

aaa gaa cat gac caa gat gtc ttg gtg ctg gag aag gtc cca gca ggg    1557
Lys Glu His Asp Gln Asp Val Leu Val Leu Glu Lys Val Pro Ala Gly
        470                 475                 480

aac aga gct tct aat caa gga aac tca cag cct cag caa aag tat act    1605
Asn Arg Ala Ser Asn Gln Gly Asn Ser Gln Pro Gln Gln Lys Tyr Thr
    485                 490                 495

gtg atg tca gga aca cct gaa aaa att tta gag cat ttt cta gaa aca    1653
Val Met Ser Gly Thr Pro Glu Lys Ile Leu Glu His Phe Leu Glu Thr
500                 505                 510                 515

ata cgc ctt gag gca act tta aat gaa gca aca gat tct gtt tta aat    1701
Ile Arg Leu Glu Ala Thr Leu Asn Glu Ala Thr Asp Ser Val Leu Asn
                520                 525                 530

gac ttt att atg atg cac tgt gtt ttt atg cca aat acc cag ctt tgc    1749
Asp Phe Ile Met Met His Cys Val Phe Met Pro Asn Thr Gln Leu Cys
            535                 540                 545

ccg gca ctg gtg gcc cac tac cac gca cag cct tca caa ggt aca gaa    1797
Pro Ala Leu Val Ala His Tyr His Ala Gln Pro Ser Gln Gly Thr Glu
        550                 555                 560

cag gag aaa atg gat tat gcc ctc aac aat aag agg cga gtc atc cgc    1845
Gln Glu Lys Met Asp Tyr Ala Leu Asn Asn Lys Arg Arg Val Ile Arg
    565                 570                 575

ctg gtt cta cag tgg gct gcc atg tat gga gac ctc ctg caa gag gat    1893
Leu Val Leu Gln Trp Ala Ala Met Tyr Gly Asp Leu Leu Gln Glu Asp
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580                 585                 590                 595

gac gta tct atg gcc ttc ctg gag gag ttt tat gta tct gta tca gat     1941
Asp Val Ser Met Ala Phe Leu Glu Glu Phe Tyr Val Ser Val Ser Asp
                600                 605                 610

gat gcc cgg atg att gct gcc ctc aag gag caa ctg cca gag ttg gag     1989
Asp Ala Arg Met Ile Ala Ala Leu Lys Glu Gln Leu Pro Glu Leu Glu
            615                 620                 625

aag att gtc aag caa atc tca gaa gat gca aag gca cca caa aag aag     2037
Lys Ile Val Lys Gln Ile Ser Glu Asp Ala Lys Ala Pro Gln Lys Lys
        630                 635                 640

cac aag gtt ctt ttg caa cag ttc aat acg ggc gat gag aga gcc cag     2085
His Lys Val Leu Leu Gln Gln Phe Asn Thr Gly Asp Glu Arg Ala Gln
    645                 650                 655

aag cgc cag cct atc cgc ggc tct gat gaa gtt ctg ttt aag gtc tat     2133
Lys Arg Gln Pro Ile Arg Gly Ser Asp Glu Val Leu Phe Lys Val Tyr
660                 665                 670                 675

tgc atg gac cac acc tac aca acc att cgg gtg cca gtg gcc act tcg     2181
Cys Met Asp His Thr Tyr Thr Thr Ile Arg Val Pro Val Ala Thr Ser
                680                 685                 690

gtg aag gaa gtc atc agt gca gtt gcc gac aag ctg ggc tcc ggg gag     2229
Val Lys Glu Val Ile Ser Ala Val Ala Asp Lys Leu Gly Ser Gly Glu
            695                 700                 705

ggc ctg atc ata gtc aag atg agt tcc gga gga gaa aag gtg gtg ctc     2277
Gly Leu Ile Ile Val Lys Met Ser Ser Gly Gly Glu Lys Val Val Leu
        710                 715                 720

aaa cct aat gat gtt tca gta ttt acg acg ctc acc att aat gga cgc     2325
Lys Pro Asn Asp Val Ser Val Phe Thr Thr Leu Thr Ile Asn Gly Arg
    725                 730                 735

ctg ttt gct tgc ccg cga gag caa ttc gat tca ctg act ccc tta cca     2373
Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu Thr Pro Leu Pro
740                 745                 750                 755

gaa cag gaa ggc cca act gtt gga aca gtg gga act ttt gaa ctg atg     2421
Glu Gln Glu Gly Pro Thr Val Gly Thr Val Gly Thr Phe Glu Leu Met
                760                 765                 770

agc tcc aaa gat tta gca tac cag atg aca att tat gat tgg gaa ctc     2469
Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Ile Tyr Asp Trp Glu Leu
            775                 780                 785

ttc aac tgc gtg cat gag ctg gag cta atc tat cac aca ttt gga agg     2517
Phe Asn Cys Val His Glu Leu Glu Leu Ile Tyr His Thr Phe Gly Arg
        790                 795                 800

cat aat ttt aaa aag acc aca gca aac ttg gat ttg ttc ctg agg aga     2565
His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu Phe Leu Arg Arg
    805                 810                 815

ttt aat gaa att cag ttt tgg gtc gtc act gag atc tgc ctt tgt tct     2613
Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile Cys Leu Cys Ser
820                 825                 830                 835
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cag ctc agc aag cgt gtt cag cta tta aaa aaa ttt att aag ata gca     2661
Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Phe Ile Lys Ile Ala
                840                 845                 850

gcc cac tgt aag gag tat aaa aat ctg aat tcc ttt ttt gcc atc gtc     2709
Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe Phe Ala Ile Val
            855                 860                 865

atg gga cta agt aac att gct gtg agc cgc ttg gca cta acg tgg gag     2757
Met Gly Leu Ser Asn Ile Ala Val Ser Arg Leu Ala Leu Thr Trp Glu
        870                 875                 880

aaa ctg cca agc aag ttc aag aag ttc tat gcg gag ttt gaa agt tta     2805
Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu Phe Glu Ser Leu
    885                 890                 895

atg gac cct tca agg aac cac agg gcc tac agg ctg aca gta gct aag     2853
Met Asp Pro Ser Arg Asn His Arg Ala Tyr Arg Leu Thr Val Ala Lys
900                 905                 910                 915

ctg gaa cct cct ctc atc ccc ttc atg cct ttg ctc att aaa gat atg     2901
Leu Glu Pro Pro Leu Ile Pro Phe Met Pro Leu Leu Ile Lys Asp Met
                920                 925                 930

aca ttt act cat gag ggg aac aag acg ttc att gac aat cta gta aac     2949
Thr Phe Thr His Glu Gly Asn Lys Thr Phe Ile Asp Asn Leu Val Asn
            935                 940                 945

ttt gaa aaa atg cgc atg att gca aat acg gcc aga aca gtg aga tac     2997
Phe Glu Lys Met Arg Met Ile Ala Asn Thr Ala Arg Thr Val Arg Tyr
        950                 955                 960

tac agg agc caa ccc ttc aat cct gat gca gct caa gct aat aag aac     3045
Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln Ala Asn Lys Asn
    965                 970                 975

cat cag gat gtc cgg agt tat gta cgg caa tta aat gtg att gac aac     3093
His Gln Asp Val Arg Ser Tyr Val Arg Gln Leu Asn Val Ile Asp Asn
980                 985                 990                 995

cag aga act tta tca  cag atg tca cac aga  tta gag cct cgt cga       3138
Gln Arg Thr Leu Ser  Gln Met Ser His Arg  Leu Glu Pro Arg Arg
                1000                1005                1010

cca tag acatttcaaa tgcccaaagc aacagtttgt ctccagtcca caatctttca      3194
Pro
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aaaatgccat ttatgctact actgactgta ttgccactag agaattctac aaaacaagca   3254

aaaacacatc ctgagacacc tcagggctgc attcagctta ccagctacct agcaagagaa   3314

ggaattattt gacatgccta aagcttacac actctagctt aggaatcccc agtttgtggc   3374

taccctgttt catttccact tgtgccatct tctttgctga agtgttgaat aaggcccacg   3434

tgaagagttt ggtagaaata gccttgtcaa gagaactagc aagcccctga catttttttc   3494

taagagtgtt tatgggatga gatgtgctac aaatgggata gatttggata aaattttaac   3554
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tcaacatctt gatttggagc ctggggtttc gggaaggtgt tgaggaatct atagaagtcc 3614

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cacattatta tgaaacatta tggctgttta tgtaatttag ggaggactgg tctgtaattt 3734

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ctttcaaaga ttgtaaatag ttcattcaat caatggtata gagttattta tttgctacat 4094

aatagatact gtgccaaata attacttttt atttatttta tttagtacgt aattttgaag 4154

tacatttttt cctgttttca caattagact acatttaatg tgtaggaatt gtatgtatgt 4214

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aaaa 4278

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Met Val Ala Ala His Ala Ala His Ser Ser Ser Ser Ala Glu Trp Ile
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Ala Cys Leu Asp Lys Arg Pro Leu Glu Arg Ser Ser Glu Asp Val Asp
20 25 30

Ile Ile Phe Thr Arg Leu Lys Glu Val Lys Ala Phe Glu Lys Phe His
35 40 45

Pro Asn Leu Leu His Gln Ile Cys Leu Cys Gly Tyr Tyr Glu Asn Leu
50 55 60

Glu Lys Gly Ile Thr Leu Phe Arg Gln Gly Asp Ile Gly Thr Asn Trp
65 70 75 80

Tyr Ala Val Leu Ala Gly Ser Leu Asp Val Lys Val Ser Glu Thr Ser
85 90 95

Ser His Gln Asp Ala Val Thr Ile Cys Thr Leu Gly Ile Gly Thr Ala
100 105 110

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Phe Gly Glu Ser Ile Leu Asp Asn Thr Pro Arg His Ala Thr Ile Val
        115                 120                 125

Thr Arg Glu Ser Ser Glu Leu Leu Arg Ile Glu Gln Lys Asp Phe Lys
    130                 135                 140

Ala Leu Trp Glu Lys Tyr Arg Gln Tyr Met Ala Gly Leu Leu Ala Pro
145                 150                 155                 160

Pro Tyr Gly Val Met Glu Thr Gly Ser Asn Asn Asp Arg Ile Pro Asp
                165                 170                 175

Lys Glu Asn Thr Pro Leu Ile Glu Pro His Val Pro Leu Arg Pro Ala
            180                 185                 190

Asn Thr Ile Thr Lys Val Pro Ser Glu Lys Ile Leu Arg Ala Gly Lys
        195                 200                 205

Ile Leu Arg Asn Ala Ile Leu Ser Arg Ala Pro His Met Ile Arg Asp
    210                 215                 220

Arg Lys Tyr His Leu Lys Thr Tyr Arg Gln Cys Cys Val Gly Thr Glu
225                 230                 235                 240

Leu Val Asp Trp Met Met Gln Gln Thr Pro Cys Val His Ser Arg Thr
                245                 250                 255

Gln Ala Val Gly Met Trp Gln Val Leu Leu Glu Asp Gly Val Leu Asn
            260                 265                 270

His Val Asp Gln Glu His His Phe Gln Asp Lys Tyr Leu Phe Tyr Arg
        275                 280                 285

Phe Leu Asp Asp Glu His Glu Asp Ala Pro Leu Pro Thr Glu Glu Glu
    290                 295                 300

Lys Lys Glu Cys Asp Glu Glu Leu Gln Asp Thr Met Leu Leu Leu Ser
305                 310                 315                 320

Gln Met Gly Pro Asp Ala His Met Arg Met Ile Leu Arg Lys Pro Pro
                325                 330                 335

Gly Gln Arg Thr Val Asp Asp Leu Glu Ile Ile Tyr Glu Glu Leu Leu
            340                 345                 350
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His Ile Lys Ala Leu Ser His Leu Ser Thr Thr Val Lys Arg Glu Leu
        355                 360                 365

Ala Gly Val Leu Ile Phe Glu Ser His Ala Lys Gly Gly Thr Val Leu
    370                 375                 380

Phe Asn Gln Gly Glu Glu Gly Thr Ser Trp Tyr Ile Ile Leu Lys Gly
385                 390                 395                 400

Ser Val Asn Val Val Ile Tyr Gly Lys Gly Val Val Cys Thr Leu His
                405                 410                 415

Glu Gly Asp Asp Phe Gly Lys Leu Ala Leu Val Asn Asp Ala Pro Arg
            420                 425                 430

Ala Ala Ser Ile Val Leu Arg Glu Asp Asn Cys His Phe Leu Arg Val
        435                 440                 445

Asp Lys Glu Asp Phe Asn Arg Ile Leu Arg Asp Val Glu Ala Asn Thr
    450                 455                 460

Val Arg Leu Lys Glu His Asp Gln Asp Val Leu Val Leu Glu Lys Val
465                 470                 475                 480

Pro Ala Gly Asn Arg Ala Ser Asn Gln Gly Asn Ser Gln Pro Gln Gln
                485                 490                 495

Lys Tyr Thr Val Met Ser Gly Thr Pro Glu Lys Ile Leu Glu His Phe
            500                 505                 510

Leu Glu Thr Ile Arg Leu Glu Ala Thr Leu Asn Glu Ala Thr Asp Ser
        515                 520                 525

Val Leu Asn Asp Phe Ile Met Met His Cys Val Phe Met Pro Asn Thr
    530                 535                 540

Gln Leu Cys Pro Ala Leu Val Ala His Tyr His Ala Gln Pro Ser Gln
545                 550                 555                 560

Gly Thr Glu Gln Glu Lys Met Asp Tyr Ala Leu Asn Asn Lys Arg Arg
                565                 570                 575

Val Ile Arg Leu Val Leu Gln Trp Ala Ala Met Tyr Gly Asp Leu Leu
            580                 585                 590

Gln Glu Asp Asp Val Ser Met Ala Phe Leu Glu Glu Phe Tyr Val Ser
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595 600 605

Val Ser Asp Asp Ala Arg Met Ile Ala Ala Leu Lys Glu Gln Leu Pro
610 615 620

Glu Leu Glu Lys Ile Val Lys Gln Ile Ser Glu Asp Ala Lys Ala Pro
625 630 635 640

Gln Lys Lys His Lys Val Leu Leu Gln Gln Phe Asn Thr Gly Asp Glu
645 650 655

Arg Ala Gln Lys Arg Gln Pro Ile Arg Gly Ser Asp Glu Val Leu Phe
660 665 670

Lys Val Tyr Cys Met Asp His Thr Tyr Thr Thr Ile Arg Val Pro Val
675 680 685

Ala Thr Ser Val Lys Glu Val Ile Ser Ala Val Ala Asp Lys Leu Gly
690 695 700

Ser Gly Glu Gly Leu Ile Ile Val Lys Met Ser Ser Gly Gly Glu Lys
705 710 715 720

Val Val Leu Lys Pro Asn Asp Val Ser Val Phe Thr Thr Leu Thr Ile
725 730 735

Asn Gly Arg Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu Thr
740 745 750

Pro Leu Pro Glu Gln Glu Gly Pro Thr Val Gly Thr Val Gly Thr Phe
755 760 765

Glu Leu Met Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Ile Tyr Asp
770 775 780

Trp Glu Leu Phe Asn Cys Val His Glu Leu Glu Leu Ile Tyr His Thr
785 790 795 800

Phe Gly Arg His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu Phe
805 810 815

Leu Arg Arg Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile Cys
820 825 830

Leu Cys Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Phe Ile
835 840 845

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Lys Ile Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe Phe
    850                 855                 860

Ala Ile Val Met Gly Leu Ser Asn Ile Ala Val Ser Arg Leu Ala Leu
865                 870                 875                 880

Thr Trp Glu Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu Phe
                885                 890                 895

Glu Ser Leu Met Asp Pro Ser Arg Asn His Arg Ala Tyr Arg Leu Thr
            900                 905                 910

Val Ala Lys Leu Glu Pro Pro Leu Ile Pro Phe Met Pro Leu Leu Ile
        915                 920                 925

Lys Asp Met Thr Phe Thr His Glu Gly Asn Lys Thr Phe Ile Asp Asn
    930                 935                 940

Leu Val Asn Phe Glu Lys Met Arg Met Ile Ala Asn Thr Ala Arg Thr
945                 950                 955                 960

Val Arg Tyr Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln Ala
                965                 970                 975

Asn Lys Asn His Gln Asp Val Arg Ser Tyr Val Arg Gln Leu Asn Val
            980                 985                 990

Ile Asp Asn Gln Arg Thr Leu Ser  Gln Met Ser His Arg  Leu Glu Pro
        995                 1000                1005

Arg Arg  Pro
    1010
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agtaaccgtt caca atg ggc agc tcc cgg ctg agg gtc ttt gac cct cat      110
                Met Gly Ser Ser Arg Leu Arg Val Phe Asp Pro His
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                1               5                  10
ttg gag agg aaa gat tcc gcc gcg gcg ctc tca gac cga gag ctg ccc      158
Leu Glu Arg Lys Asp Ser Ala Ala Ala Leu Ser Asp Arg Glu Leu Pro
        15                  20                  25

ttg cct acc ttc gat gtg cct tat ttc aaa tac atc gac gag gag gat      206
Leu Pro Thr Phe Asp Val Pro Tyr Phe Lys Tyr Ile Asp Glu Glu Asp
    30                  35                  40

gag gac gat gaa tgg agc agc cgc tcg cag tct tcc acc gag gat gac      254
Glu Asp Asp Glu Trp Ser Ser Arg Ser Gln Ser Ser Thr Glu Asp Asp
45                  50                  55                  60

tca gtg gac tct ctg ctc tct gac aga tat gtg gtg gtg tcc ggg acc      302
Ser Val Asp Ser Leu Leu Ser Asp Arg Tyr Val Val Val Ser Gly Thr
                65                  70                  75

ccg gag aag att ttg gag cac ctt ttg aat gac ttg cac ctg gaa gaa      350
Pro Glu Lys Ile Leu Glu His Leu Leu Asn Asp Leu His Leu Glu Glu
            80                  85                  90

gtc cag gac aaa gaa aca gag acc ctc ctg gat gac ttc ctt ctc acg      398
Val Gln Asp Lys Glu Thr Glu Thr Leu Leu Asp Asp Phe Leu Leu Thr
        95                  100                 105

tac act gtc ttc atg aca act gat gac ttg tgc cag gct ctg tta agg      446
Tyr Thr Val Phe Met Thr Thr Asp Asp Leu Cys Gln Ala Leu Leu Arg
    110                 115                 120

cac tat tct gct aag aag tat caa ggc aaa gag gaa aac tca gat gtt      494
His Tyr Ser Ala Lys Lys Tyr Gln Gly Lys Glu Glu Asn Ser Asp Val
125                 130                 135                 140

ccg cgt agg aaa cgt aaa gtc ttg cat ctt gtt tcc cag tgg att gct      542
Pro Arg Arg Lys Arg Lys Val Leu His Leu Val Ser Gln Trp Ile Ala
                145                 150                 155

ctg tac aaa gac tgg tta cct gaa gat gaa cat tca aaa atg ttt tta      590
Leu Tyr Lys Asp Trp Leu Pro Glu Asp Glu His Ser Lys Met Phe Leu
            160                 165                 170

aag acc ata tat agg aat gta ctg gat gat gtt tat gaa tat cca ata      638
Lys Thr Ile Tyr Arg Asn Val Leu Asp Asp Val Tyr Glu Tyr Pro Ile
        175                 180                 185

ctt gaa aaa gaa ttg aaa gaa ttt caa aag ata ctt gga atg cac cgt      686
Leu Glu Lys Glu Leu Lys Glu Phe Gln Lys Ile Leu Gly Met His Arg
    190                 195                 200

cgt cac act gta gat gaa tat tca cca caa aaa aag aat aaa gcc ctt      734
Arg His Thr Val Asp Glu Tyr Ser Pro Gln Lys Lys Asn Lys Ala Leu
205                 210                 215                 220

ttc cac caa ttc agt ctt aag gag aac tgg ctc cag cat aga gga act      782
Phe His Gln Phe Ser Leu Lys Glu Asn Trp Leu Gln His Arg Gly Thr
                225                 230                 235

gtg act gaa acg gag gaa att ttc tgc cac gtg tat ata aca gag cac      830
Val Thr Glu Thr Glu Glu Ile Phe Cys His Val Tyr Ile Thr Glu His
            240                 245                 250
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tcc tat gtc agt gtg aag gca aaa gtt tcc agt ata gcc caa gag att      878
Ser Tyr Val Ser Val Lys Ala Lys Val Ser Ser Ile Ala Gln Glu Ile
        255                 260                 265

ctg ctc tgc agc cag ctg ggc aag cga gtg cag ctg gtg aaa aaa ttc      926
Leu Leu Cys Ser Gln Leu Gly Lys Arg Val Gln Leu Val Lys Lys Phe
    270                 275                 280

atc aaa att gcg gct cac tgc aaa gcc cag aga aac ctg aat tct ttc      974
Ile Lys Ile Ala Ala His Cys Lys Ala Gln Arg Asn Leu Asn Ser Phe
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ttt gcc att gtg atg ggt ctc aac act gct tct gtc agt cga ctg tcg     1022
Phe Ala Ile Val Met Gly Leu Asn Thr Ala Ser Val Ser Arg Leu Ser
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cag acc tgg gag aaa atc cct ggg aag ttt aag aaa ctt ttc tct gaa     1070
Gln Thr Trp Glu Lys Ile Pro Gly Lys Phe Lys Lys Leu Phe Ser Glu
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ctt gaa agt tta aca gat cct tcc cta aat cac aaa gcc tac aga gat     1118
Leu Glu Ser Leu Thr Asp Pro Ser Leu Asn His Lys Ala Tyr Arg Asp
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gca ttc aaa aag atg aag cca cca aaa atc cct ttc gtg ccc tta ttg     1166
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ctt aaa gat gta aca ttt att cat gaa gga aat aaa act ttt ttg gat     1214
Leu Lys Asp Val Thr Phe Ile His Glu Gly Asn Lys Thr Phe Leu Asp
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aat ctt gtc aat ttt gaa aag ctg cat atg atc gca gac act gtc cga     1262
Asn Leu Val Asn Phe Glu Lys Leu His Met Ile Ala Asp Thr Val Arg
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acc ctg aga cac tgc agg act aac cag ttt ggt gac ctg tct cca aaa     1310
Thr Leu Arg His Cys Arg Thr Asn Gln Phe Gly Asp Leu Ser Pro Lys
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gag cat caa gag tta aag tcc tat gtt aat cac ctg tat gtc att gac     1358
Glu His Gln Glu Leu Lys Ser Tyr Val Asn His Leu Tyr Val Ile Asp
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agc cag cag gct ctg ttt gag ctc tca cac agg atc gag cct cgg gtg     1406
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35 40 45

Trp Ser Ser Arg Ser Gln Ser Ser Thr Glu Asp Asp Ser Val Asp Ser
50 55 60

Leu Leu Ser Asp Arg Tyr Val Val Val Ser Gly Thr Pro Glu Lys Ile
65 70 75 80

Leu Glu His Leu Leu Asn Asp Leu His Leu Glu Glu Val Gln Asp Lys
85 90 95

Glu Thr Glu Thr Leu Leu Asp Asp Phe Leu Leu Thr Tyr Thr Val Phe
100 105 110

Met Thr Thr Asp Asp Leu Cys Gln Ala Leu Leu Arg His Tyr Ser Ala
115 120 125

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130 135 140

Arg Lys Val Leu His Leu Val Ser Gln Trp Ile Ala Leu Tyr Lys Asp
145 150 155 160

Trp Leu Pro Glu Asp Glu His Ser Lys Met Phe Leu Lys Thr Ile Tyr
165 170 175

Arg Asn Val Leu Asp Asp Val Tyr Glu Tyr Pro Ile Leu Glu Lys Glu
180 185 190

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Leu Lys Glu Phe Gln Lys Ile Leu Gly Met His Arg Arg His Thr Val
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Asp Glu Tyr Ser Pro Gln Lys Lys Asn Lys Ala Leu Phe His Gln Phe
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Ser Leu Lys Glu Asn Trp Leu Gln His Arg Gly Thr Val Thr Glu Thr
225                 230                 235                 240

Glu Glu Ile Phe Cys His Val Tyr Ile Thr Glu His Ser Tyr Val Ser
                245                 250                 255

Val Lys Ala Lys Val Ser Ser Ile Ala Gln Glu Ile Leu Leu Cys Ser
            260                 265                 270

Gln Leu Gly Lys Arg Val Gln Leu Val Lys Lys Phe Ile Lys Ile Ala
        275                 280                 285

Ala His Cys Lys Ala Gln Arg Asn Leu Asn Ser Phe Phe Ala Ile Val
    290                 295                 300

Met Gly Leu Asn Thr Ala Ser Val Ser Arg Leu Ser Gln Thr Trp Glu
305                 310                 315                 320

Lys Ile Pro Gly Lys Phe Lys Lys Leu Phe Ser Glu Leu Glu Ser Leu
                325                 330                 335

Thr Asp Pro Ser Leu Asn His Lys Ala Tyr Arg Asp Ala Phe Lys Lys
            340                 345                 350

Met Lys Pro Pro Lys Ile Pro Phe Val Pro Leu Leu Leu Lys Asp Val
        355                 360                 365

Thr Phe Ile His Glu Gly Asn Lys Thr Phe Leu Asp Asn Leu Val Asn
    370                 375                 380

Phe Glu Lys Leu His Met Ile Ala Asp Thr Val Arg Thr Leu Arg His
385                 390                 395                 400

Cys Arg Thr Asn Gln Phe Gly Asp Leu Ser Pro Lys Glu His Gln Glu
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Pro Gly Arg Asn Arg Tyr Thr Val Met Ser Gly Thr Pro Glu Lys Ile
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cta gag ctt ctg ttg gag gcc atg gga cca gat tcc agt gct cat gac      144
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cca aca gag aca ttc ctc agc gac ttc ctc ctg acc cac agg gtc ttc      192
Pro Thr Glu Thr Phe Leu Ser Asp Phe Leu Leu Thr His Arg Val Phe
    50                  55                  60

atg ccc agc gcc caa ctc tgc gct gcc ctt ctg cac cac ttc cat gtg      240
Met Pro Ser Ala Gln Leu Cys Ala Ala Leu Leu His His Phe His Val
65                  70                  75                  80

gag cct gcg ggt ggc agc gag cag gag cgc agc acc tac gtc tgc aac      288
Glu Pro Ala Gly Gly Ser Glu Gln Glu Arg Ser Thr Tyr Val Cys Asn
                85                  90                  95

aag agg cag cag atc ttg cgg ctg gtc agc cag tgg gtg gcc ctg tat      336
Lys Arg Gln Gln Ile Leu Arg Leu Val Ser Gln Trp Val Ala Leu Tyr
            100                 105                 110

ggc tcc atg ctc cac act gac cct gtg gcc acc agc ttc ctc cag aaa      384
Gly Ser Met Leu His Thr Asp Pro Val Ala Thr Ser Phe Leu Gln Lys
        115                 120                 125

ctc tca gac ctg gtg ggc agg gac acc cga ctc agc aac ctg ctg agg      432
Leu Ser Asp Leu Val Gly Arg Asp Thr Arg Leu Ser Asn Leu Leu Arg
    130                 135                 140

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Glu Gln Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn Gly Cys
145                 150                 155                 160

ggg aat gca tct cct cag atg aag gcc cgg aac ttg cct gtt tgg ctc      528
Gly Asn Ala Ser Pro Gln Met Lys Ala Arg Asn Leu Pro Val Trp Leu
                165                 170                 175

ccc aac cag gac gag ccc ctt cct ggc agc agc tgt gcc atc caa gtt      576
Pro Asn Gln Asp Glu Pro Leu Pro Gly Ser Ser Cys Ala Ile Gln Val
            180                 185                 190
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ggg gat aaa gtc ccc tat gac atc tgc cgg cca gac cac tca gtg ttg      624
Gly Asp Lys Val Pro Tyr Asp Ile Cys Arg Pro Asp His Ser Val Leu
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acc ctg cag ctg cct gtg aca gcc tcc gtg aga gag gtg atg gca gcg      672
Thr Leu Gln Leu Pro Val Thr Ala Ser Val Arg Glu Val Met Ala Ala
    210                 215                 220

ttg gcc cag gag gat ggc tgg acc aag ggg cag gtg ctg gtg aag gtc      720
Leu Ala Gln Glu Asp Gly Trp Thr Lys Gly Gln Val Leu Val Lys Val
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aat tct gca ggt gat gcc att ggc ctg cag cca gat gcc cgt ggt gtg      768
Asn Ser Ala Gly Asp Ala Ile Gly Leu Gln Pro Asp Ala Arg Gly Val
                245                 250                 255

gcc aca tct ctg ggg ctc aat gag cgt ctc ttt gtt gtc aac cca cag      816
Ala Thr Ser Leu Gly Leu Asn Glu Arg Leu Phe Val Val Asn Pro Gln
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gaa gtg cat gag ctg atc cca cac cct gac cag ctg ggg ccc act gtg      864
Glu Val His Glu Leu Ile Pro His Pro Asp Gln Leu Gly Pro Thr Val
        275                 280                 285

ggc tct gct gag ggg ctg gac ctg gtg agt gcc aag gac ctg gca ggc      912
Gly Ser Ala Glu Gly Leu Asp Leu Val Ser Ala Lys Asp Leu Ala Gly
    290                 295                 300

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Gln Leu Thr Asp His Asp Trp Ser Leu Phe Asn Ser Ile His Gln Val
305                 310                 315                 320

gag ctg atc cac tat gtg ctg ggc ccc cag cat ctg cgg gat gtc acc     1008
Glu Leu Ile His Tyr Val Leu Gly Pro Gln His Leu Arg Asp Val Thr
                325                 330                 335

acc gcc aac ctg gag cgc ttc atg cgc cgc ttc aat gag ctg cag tac     1056
Thr Ala Asn Leu Glu Arg Phe Met Arg Arg Phe Asn Glu Leu Gln Tyr
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tgg gtg gcc acc gag ctg tgt ctc tgc ccc gtg ccc ggc ccc cgg gcc     1104
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gcc atc agc cgc cta gcc cac acc tgg gag cgg ctg cct cac aaa gtc     1248
Ala Ile Ser Arg Leu Ala His Thr Trp Glu Arg Leu Pro His Lys Val
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cgg aag ctg tac tcc gcc ctc gag agg ctg ctg gat ccc tca tgg aac     1296
Arg Lys Leu Tyr Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser Trp Asn
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cac cgg gta tac cga ctg gcc ctc gcc aag ctc tcc cct cct gtc atc     1344
His Arg Val Tyr Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro Val Ile
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ccc ttc atg ccc ctt ctt ctc aaa gac atg acc ttc att cat gag gga     1392
Pro Phe Met Pro Leu Leu Leu Lys Asp Met Thr Phe Ile His Glu Gly
    450                 455                 460

aac cac aca cta gtg gag aat ctc atc aac ttt gag aag atg aga atg     1440
Asn His Thr Leu Val Glu Asn Leu Ile Asn Phe Glu Lys Met Arg Met
465                 470                 475                 480

atg gcc aga gcc gcg cgg atg ctg cac cac tgc cga agc cac aac cct     1488
Met Ala Arg Ala Ala Arg Met Leu His His Cys Arg Ser His Asn Pro
                485                 490                 495

gtg cct ctc tca cca ctc aga agc cga gtt tcc cac ctc cac gag gac     1536
Val Pro Leu Ser Pro Leu Arg Ser Arg Val Ser His Leu His Glu Asp
            500                 505                 510

agc cag gtg gcg agg att tcc aca tgc tcg gag cag tcc ctg agc acc     1584
Ser Gln Val Ala Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu Ser Thr
        515                 520                 525

cgg agt cca gcc agc acc tgg gct tat gtc cag cag ctg aag gtc att     1632
Arg Ser Pro Ala Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys Val Ile
    530                 535                 540

gac aac cag cgg gaa ctc tcc cgc ctc tcc cga gag ctg gag cca tga     1680
Asp Asn Gln Arg Glu Leu Ser Arg Leu Ser Arg Glu Leu Glu Pro
545                 550                 555

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Pro Thr Glu Thr Phe Leu Ser Asp Phe Leu Leu Thr His Arg Val Phe
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Met Pro Ser Ala Gln Leu Cys Ala Ala Leu Leu His His Phe His Val
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Glu Pro Ala Gly Gly Ser Glu Gln Glu Arg Ser Thr Tyr Val Cys Asn
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Lys Arg Gln Gln Ile Leu Arg Leu Val Ser Gln Trp Val Ala Leu Tyr
100 105 110

Gly Ser Met Leu His Thr Asp Pro Val Ala Thr Ser Phe Leu Gln Lys
115 120 125

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Glu Gln Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn Gly Cys
145 150 155 160

Gly Asn Ala Ser Pro Gln Met Lys Ala Arg Asn Leu Pro Val Trp Leu
165 170 175

Pro Asn Gln Asp Glu Pro Leu Pro Gly Ser Ser Cys Ala Ile Gln Val
180 185 190

Gly Asp Lys Val Pro Tyr Asp Ile Cys Arg Pro Asp His Ser Val Leu
195 200 205

Thr Leu Gln Leu Pro Val Thr Ala Ser Val Arg Glu Val Met Ala Ala
210 215 220

Leu Ala Gln Glu Asp Gly Trp Thr Lys Gly Gln Val Leu Val Lys Val
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Asn Ser Ala Gly Asp Ala Ile Gly Leu Gln Pro Asp Ala Arg Gly Val
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Glu Val His Glu Leu Ile Pro His Pro Asp Gln Leu Gly Pro Thr Val

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Gln Leu Thr Asp His Asp Trp Ser Leu Phe Asn Ser Ile His Gln Val
305                 310                 315                 320

Glu Leu Ile His Tyr Val Leu Gly Pro Gln His Leu Arg Asp Val Thr
                325                 330                 335

Thr Ala Asn Leu Glu Arg Phe Met Arg Arg Phe Asn Glu Leu Gln Tyr
            340                 345                 350

Trp Val Ala Thr Glu Leu Cys Leu Cys Pro Val Pro Gly Pro Arg Ala
        355                 360                 365

Gln Leu Leu Arg Lys Phe Ile Lys Leu Ala Ala His Leu Lys Glu Gln
    370                 375                 380

Lys Asn Leu Asn Ser Phe Phe Ala Val Met Phe Gly Leu Ser Asn Ser
385                 390                 395                 400

Ala Ile Ser Arg Leu Ala His Thr Trp Glu Arg Leu Pro His Lys Val
                405                 410                 415

Arg Lys Leu Tyr Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser Trp Asn
            420                 425                 430

His Arg Val Tyr Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro Val Ile
        435                 440                 445

Pro Phe Met Pro Leu Leu Leu Lys Asp Met Thr Phe Ile His Glu Gly
    450                 455                 460

Asn His Thr Leu Val Glu Asn Leu Ile Asn Phe Glu Lys Met Arg Met
465                 470                 475                 480

Met Ala Arg Ala Ala Arg Met Leu His His Cys Arg Ser His Asn Pro
                485                 490                 495

Val Pro Leu Ser Pro Leu Arg Ser Arg Val Ser His Leu His Glu Asp
            500                 505                 510

Ser Gln Val Ala Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu Ser Thr
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Arg Ser Pro Ala Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys Val Ile
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Asp Asn Gln Arg Glu Leu Ser Arg Leu Ser Arg Glu Leu Glu Pro
545 550 555

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2006/001903

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [X] filed together with the international application in electronic form

   [ ] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/001903

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K45/00 A61P9/00 A61P9/04 A61K31/335 A61K31/7076
A01K67/02 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/24768 A (MASSACHUSETTS INSTITUTE OF TECHNOLOGY; KAWASAKI, HIROAKI; GRAYBIEL, AN) 4 May 2000 (2000-05-04)	13-15, 20,23
A	page 27, line 13 - page 28, line 7 page 44, line 17 - line 26 page 29, line 29 - page 30, line 15	1-12, 15-19, 21,22
A	WO 00/10603 A (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 2 March 2000 (2000-03-02) claims 16-8	1-23

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2006	09/06/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Böhmerova, E

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/001903

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WHALEN ERIN J ET AL: "Blockade of beta-adrenoceptors enhances cAMP signal transduction in vivo" EUROPEAN JOURNAL OF PHARMACOLOGY, vol. 355, no. 2-3, 21 August 1998 (1998-08-21), pages 189-193, XP002382243 ISSN: 0014-2999 abstract page 191, right-hand column, last paragraph - page 192, left-hand column, paragraph 1	1-23
P,X	US 6 987 004 B1 (KAWASAKI HIROAKI ET AL) 17 January 2006 (2006-01-17) column 32, line 39 - line 66 column 33, line 41 - line 56 sequence 59	13,14
P,X	OSADCHII OLEG ET AL: "Inotropic responses to phosphodiesterase inhibitors in cardiac hypertrophy in rats" EUROPEAN JOURNAL OF PHARMACOLOGY, vol. 514, no. 2-3, May 2005 (2005-05), pages 201-208, XP004904401 ISSN: 0014-2999 abstract page 204, left-hand column, last paragraph - right-hand column, paragraph 1; figure 4	1-12
T	MOREL ERIC ET AL: "cAMP-binding protein Epac induces cardiomyocyte hypertrophy" CIRCULATION RESEARCH, vol. 97, no. 12, December 2005 (2005-12), pages 1296-1304, XP009066625 ISSN: 0009-7330 the whole document	1-23

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Information on patent family members

International application No
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WO 0010603 A	02-03-2000	CA 2341558 A1	02-03-2000
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